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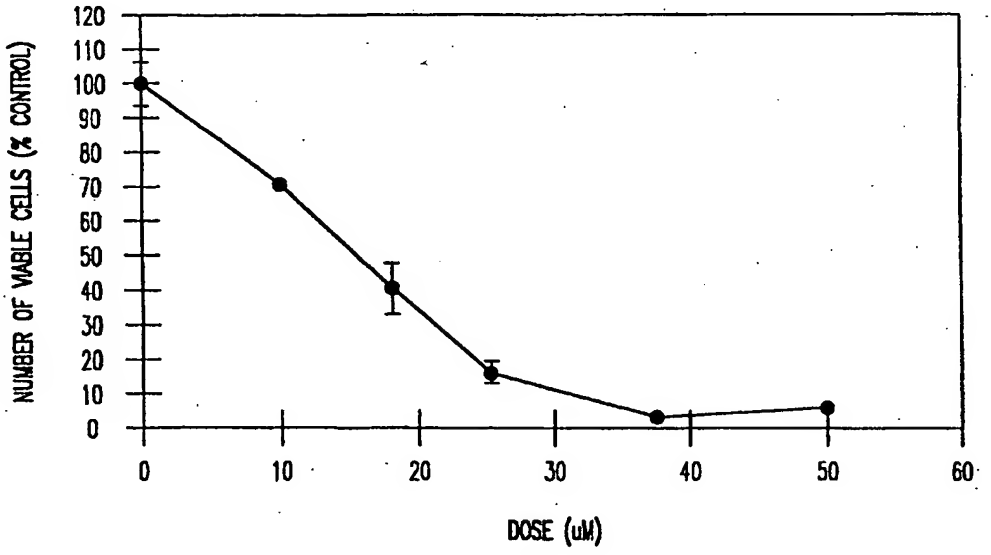

(57) Abstract

The present invention provides methods for the use of derivatives of Vitamin E (tocopherol and tocotrienol), cholesterol, taxol and betulinic acid as antitumor agents for the treatment of and prevention of cancers of the liver, lung, colon, prostate and breast as well as melanomas and leukemias.

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ANTI-TUMOR ACTIVITY OF VITAMIN E, CHOLESTEROL, TAXOL AND BETULINIC ACID DERIVATIVES

DESCRIPTION

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention generally relates to the use of derivatives of Vitamin E (tocopherol and tocotrienol), cholesterol, taxol and betulinic acid as antitumor agents for the treatment of and prevention of cancers of the liver, lung, colon, prostate and breast as well as melanomas and leukemias.

Background Description

The antitumor effects of α-tocopheryl hemisuccinate, free acid (TS) have been reported in numerous tumor cell types (1-5). Although it was previously believed that the effects of this compound were due to the enzymatic release of α-tocopherol (Vitamin E) by esterases in the cell, recent studies have shown that the antitumor effects of TS are instead due to the intact molecule (4, 6). In support of the intact TS molecule as the active cytotoxic agent, the non-hydrolyzable ether derivative of TS, tocopheryoxybutyric acid (TSE) has also been shown to possess antitumor activity against leukemia (6) and breast cancer cells (10). Further, it has been shown that the antitumor effects of TS are not exhibited by unesterified α-tocopherol (7). A related compound, cholesteryl hemisuccinate (CS) appears to have antitumor properties similar to those observed for TS (6, 8 and 9). However, the antitumor effects of CS and its ether derivative cholesteryloxybutyric acid (CSE) have been tested in only murine leukemia cells (6) and breast cancer cells (10).

Though the administration of TS, TSE, CS and CSE have been shown to be cytotoxic for several tumor cell types such as leukemias and breast (hormone-dependent), the activity of related compounds in other tumor cell types is unknown and cannot be predicted from previous studies. For example, TS, TSE, CS or CSE treatments do not appear to adversely affect some cell types such as murine-derived bone marrow cells (6) and numerous other normal cell types (2,3,13).

It is thus of interest to 1) identify and develop new derivatives of Vitamin E, cholesterol, taxol and betulinic acid which may be effective anti-tumor agents, and 2) determine the range of cancer types that can be treated with these derivatives.

SUMMARY

It is an object of this invention to provide a method of use of derivatives of Vitamin E (tocopherol and tocotrienol), cholesterol, taxol and betulinic acid as anti-tumor agents. In

particular, it is an object of this invention to provide a method of use as anti-tumor agents for the following derivatives: d-α-Tocopheryl succinate tris salt, d-α-Tocopheryl methyl succinate, d-α-Tocopheryloxybutyric acid, d-δ-Tocopheryl succinate, d-γ-Tocopheryl succinate tris salt, d-α-Tocopheryl 2,2-dimethyl glutarate, d-α-Tocopheryl 2,2-dimethyl succinate, d-Tocopheryl γ-dimethyl aminobutyrate methiodide, d-α-Tocopheryl γ-dimethyl aminobutyrate, d-α-Tocopheryl succinate polyethylene glycol 1000, tocotrienol rich fraction succinate ester, tocotrienol rich fraction succinate tris salt, d-α-Tocopheryloxybutyric acid polyethylene glycol 1000, γ-Tocotrienol succinate, cholesteryl succinate tris salt, cholesteryl succinate polyethylene glycol 1000, γ-cholesteryloxybutyric acid tris salt, cholesterol hydrogen phthalate, taxol monosuccinate tris salt, taxol disuccinate tris salt, and betulinic acid succinate. The derivatives of the present invention may be used alone or in combination with one another, or with other antitumor agents.

It is an object of the present invention to provide a method of use of the PEG esters of vitamin E, cholesterol, taxol and betulinic acid as antitumor agents.

In addition, it is an object of the present invention to provide a method of use of the tris salts of derivatives of tocopherol, cholesterol, taxol and betulinic acid as antitumor agents.

Further, it is an object of the present invention to provide five new compounds which may be utilized as antitumor agents in the practice of the methods of the present invention: d-α-tocopheryl 2,2-dimethylglutarate; d-α--tocopheryl 2,2 dimethyglutarate tris salt; d-α-tocopheryl β-(N,N-dimethylamino)ethyl ether (free base); d-α-tocopheryl β-(N,N-dimethylamino)ethyl ether oxalate salt; and d-α-tocopheryl β-N,N-(dimethylamino)ethyl ether methiodide.

ABBREVIATIONS

α-TS	d-α-Tocopheryl succinate
α-TS-T	d-α-Tocopheryl succinate tris salt
α-T-MS	d-α-Tocopheryl methyl succinate
TSE	d-α-Tocopheryloxybutyric acid
TSE-T	d-α-Tocopheryloxybutyric acid tris salt
δ-TS	d-δ-Tocopheryl succinate
γ-TS-T	d-γ-Tocopheryl succinate tris salt
2,2-Dim-TG	d-α-Tocopheryl 2,2-dimethyl glutarate
2,2-Dim-TS	d-α-Tocopheryl 2,2-dimethyl succinate
T-DMAB-Q	d-Tocopheryl γ-dimethyl aminobutyrate methiodide
T-DMAB-T	d-α-Tocopheryl γ-dimethyl aminobutyrate
T-DMAE ether	d-α-Tocopheryl-β-N,N-dimethylamino) ethyl ether

δ-T	d-δ-Tocopherol
γ-T	d-γ-Tocopherol
T-P-Na_2	dl-α-Tocopheryl phosphate disodium salt
TS-PEG	d-α-Tocopheryl succinate polyethylene glycol 1000
TSE-PEG	d-α-Tocopheryloxybutyric acid polyethylene glycol 1000
PEG 1000	polyethylene glycol 1000
TRF	Tocotrienol rich fraction
TRF-S	Tocotrienol rich fraction succinate ester
TRF-S-T	Tocotrienol rich fraction succinate tris salt
Rice-B-oil	Rice bran oil tocol concentrate
Palm oil	Palm oil tocol concentrate
γ-T3	γ-Tocotrienol
γ-T3-S	γ-Tocotrienol succinate
γ-T3-S-tris	γ-Tocotrienol succinate tris salt
γ-T3 acetate	γ-Tocotrienol acetate
CS-tris	Cholesteryl succinate tris salt
CS-PEG	Cholesteryl succinate polyethylene glycol 1000
γ-CSE-tris	γ-cholesteryloxybutyric acid tris salt
Choles-h-p	Cholesteryl hydrogen phthalate
Btl-acid	Betulinic acid
Btl-acid succinate	Betulinic acid succinate
Taxol-S-tris	Taxol monosuccinate tris salt
Taxol-DS-tris	Taxol disuccinate tris salt

DETAILED DESCRIPTION OF THE DRAWINGS

Figure 1A-1F. Effect of indicated compounds on the growth of Hep3B liver cells. **Fig.1A:** δ-TS (●) and αTS (○); **Fig.1B:** TSE; **Fig.1C:** γ-TS-tris; **Fig.1D:** α-T-MS (●) and TS (○); **Fig.1E:** T-DMAB-Q; **Fig.1F:** δ-T.

Figure 2A-2F. Effect of indicated compounds on the growth of Hep3B liver cells. **Fig.2A**: γ-T; **Fig.2B**: PEG 1000; **Fig.2C**: T-P-Na_2; **Fig.2D**: TS-PEG; **Fig.2E**: TSE-PEG; **Fig.2F**: TRF.

Figure 3A-3F. Effect of indicated compounds on the growth of Hep3B liver cells. **Fig.3A**: TRF-S-T; **Fig.3B**: TRF-S; **Fig.3C**: Rice bran oil; **Fig.3D**: Palm oil; **Fig.3E**: γ-T3; **Fig.3F**: γ-T3 succinate.

Figure 4A-4F. Effect of indicated compounds on the growth of Hep3B liver cells. **Fig.4A**: γ-T3 acetate; **Fig.4B**: CS-tris (●) and CSE-tris (○); **Fig.4C**: CS-PEG; **Fig.4D**: Choles-h-p; **Fig.4E**: Btl-acid succinate; **Fig.4F**: Btl-acid.

Figure 5A-5F. Effect of indicated compounds on the growth of Hep3B liver cells. **Fig.5A**: TS-tris; **Fig.5B**: TSE-tris; **Fig.5C**: CSE-tris; **Fig.5D**: 2,2-Dim-TS; **Fig.5E**: T-DMAB-Q; **Fig.5F**: T-DMAB-T.

Figure 6. Effect of DMAB-ether on the growth of Hep3B liver cells.

Figure 7A-7F. Effect of indicated compounds on the growth of Du145 prostate cancer cells. **Fig.7A**: αT-MS (○) and αTS (▼); **Fig.7B**: TSE; **Fig.7C**: T-DMAB-Q; **Fig.7D**: PEG 1000; **Fig.7E**: TS-PEG; **Fig.7F**: TSE-PEG.

Figure 8A-8F. Effect of indicated compounds on the growth of Du145 prostate cancer cells. **Fig.8A**: TRF; **Fig.8B**: TRF-S; **Fig.8C**: γ-T3; **Fig.8D**: γ-T3-S; **Fig.8E**: CS-tris; **Fig.8F**: CS-PEG.

Figure 9A-9D. Effect of indicated compounds on the growth of Du145 prostate cancer cells. **Fig.9A**: TS-tris; **Fig.9B**: 2,2-Dim-TS; **Fig.9C**: T-DMAB-T; **Fig.9D**: choles-h-p.

Figure 10A-10F. Effect of indicated compounds on the growth of NIH-H69 small cell lung cancer cells. **Fig.10A**: α-T-MS; **Fig.10B**: γ-TS-tris; **Fig.10C**: δ-TS; **Fig.10D**: αTS; **Fig.10E**: TSE; **Fig.10F**: PEG 1000.

Figure 11A-11F. Effect of indicated compounds on the growth of NIH-H69 small cell lung cancer cells. **Fig.11A**: δ-T; **Fig.11B**: T-P-Na_2; **Fig.11C**: γ-T; **Fig.11D**: T-DMAB-Q; **Fig.11E**: TSE-PEG; **Fig.11F**: TS-PEG.

Figure 12A-12E. Effect of indicated compounds on the growth of NIH-H69 small cell lung cancer cells. **Fig.12A**: TRF-S-T; **Fig.12B**: TRF-S; **Fig.12C**: TRF; **Fig.12D**: Rice bran oil; **Fig.12E**: Palm oil.

Figure 13A-13F. Effect of indicated compounds on the growth of NIH-H69 small cell lung cancer cells. **Fig.13A**: γ-T3; **Fig.13B**: γ-T3-S; **Fig.13C**: γ-T3 acetate; **Fig.13D**: CSE-tris; **Fig.13E**: CS-PEG; **Fig.13F**: CS-tris.

Figure 14A-14E. Effect of indicated compounds on the growth of NIH-H69 small cell lung cancer cells. **Fig.14A**: TS-tris; **Fig.14B**: TSE; **Fig.14C**: T-DMAB-T; **Fig.14D**: choles-h-p; **Fig.14E**: 2,2-Dim-TS.

Figure 15A-15F. Effect of indicated compounds on the growth of HT29 colon cancer cells. **Fig.15A**: α-T-MS; **Fig.15B**: TS; **Fig.15C**: 2,2-Dim-TG; **Fig.15D**: TSE; **Fig.15E**: PEG 1000; **Fig.15F**: TS-PEG.

Figure 16A-16E. Effect of indicated compounds on the growth of HT29 colon cancer cells. **Fig.16A**:TRF (●) and TRF-S (○); **Fig.16B**: γ-T3 (●) and γ-T3-S (○); **Fig.16C**: CSE-PEG; **Fig.16D**: CS-PEG; **Fig.16E**: CS-tris.

Figure 17A-17F. Effect of indicated compounds on the growth of HT29 colon cancer cells. **Fig.17A**: TS-tris; **Fig.17B**: TSE-tris; **Fig.17C**: CSE-tris; **Fig.17D**: T-DMAB-Q **Fig.17E**: T-DMAB-T; **Fig.17F**: choles-h-p.

Figure 18. Effect of indicated compounds on the growth of HT29 colon cancer cells. **Fig. 18A:** TSE-PEG; **Fig. 18B:** 2,2-Dim-TS.

Figure 19A-19E. Effect of indicated compounds on the growth of MCF-7 breast cancer cells. **Fig.19A**: PEG-1000; **Fig.19B**: α-T-MS (●) and TS (○); **Fig.19C**: TSE; **Fig.19D**: TRF-S (●) and TRF (○); **Fig.19E**: TSE-PEG (●) and TS-PEG (○).

Figure 20A-20C. Effect of indicated compounds on the growth of MCF-7 breast cancer cells. **Fig.20A**: CS-tris; **Fig.20B**:CS-PEG; **Fig.20C**: γ-T3-S (●) and γ-T3 (○).

Figure 21A-21D. Effect of indicated compounds on the growth of MDA-231 breast cancer cells. **Fig.21A**: TRF-tris; **Fig.21B**: TRF; **Fig.21C**: TS-tris; **Fig.21D**: TS.

Figure 22A-2D. Effect of indicated compounds on the growth of MDA-231 breast cancer cells. **Fig.22A**: TSE; **Fig.22B**: TS-PEG; **Fig.22C**: 2,2-Dim-TS; **Fig.22D**: T-DMAB-T.

Figure 23A-23D. Effect of indicated compounds on the growth of HL-60 leukemia cancer cells. **Fig.23A**: γ-T3 (●), γ-T3-S (○), γ-T3-S-tris (▼) and γ-T3-acetate (▽); **Fig.23B**: TRF (●), TRF-S (○) and TRF-S-T (▼); **Fig.23C**: Choles-h-p; **Fig.23D**: T-DMAB-T.

Figure 24A-24D. Effect of indicated compounds on the growth of A375 cutaneous melanoma cancer cells. **Fig.24A**: α-T-MS (●) and TS (○); **Fig.24B**: TSE; **Fig.24C**: TRF (●) and TRF-S (○); **Fig.24D**: TS-PEG (●) and TSE-PEG (○).

Figure 25A-25C. Effect of indicated compounds on the growth of A375 cutaneous melanoma cancer cells. **Fig.25A**: CS-PEG; **Fig.25B**:CS-tris; **Fig.25C**: γ-T3-S (●) and γ-T3 (○).

Figure 26A-26D. Effect of indicated compounds on the growth of A375 cutaneous melanoma cancer cells. **Fig.26A**: TS-tris; **Fig.26B**: TSE-tris; **Fig.26C**: 2,2-Dim-TS; **Fig.26D**: T-DMAB-T.

Figure 27A-27E. Effect of indicated compounds on the growth of OCM1 ocular melanoma cancer cells. **Fig.27A**: α-T-MS (○) and TS (▼); **Fig.27B**: TSE; **Fig.27C**: 2,2-Dim-TG; **Fig.27D**: TS-PEG; **Fig.27E**: TSE-PEG.

Figure 28A-28F. Effect of indicated compounds on the growth of OCM1 ocular melanoma cancer cells. **Fig.28A**: T-DMAB-Q; **Fig.28B**: PEG 1000; **Fig.28C**: γ-T3-S; **Fig.28D**: γ-T3; **Fig.28E**: TRF; **Fig.28F**: TRF-S.

Figure 29A-29F. Effect of indicated compounds on the growth of OCM1 ocular melanoma cancer cells. **Fig.29A**: 2,2-Dim-TS; **Fig.29B**: Btl-acid (●) and Btl-acid succinate (○); **Fig.29C**: T-DMAB-T; **Fig.29D**: CS-tris; **Fig.29E**: CS-PEG; **Fig.29F**: Choles-h-p.

Figure 30A-30D. Effect of indicated compounds on the growth of OCM1 ocular melanoma cancer cells. **Fig.30A**: TS-tris; **Fig.30B**: TSE-tris; **Fig.30C**: T-DMAB-T; **Fig.30D**: T-DMAB-Q.

Figure 31A-31C. Effect of indicated compounds on the growth of OCM1 ocular melanoma cancer cells. **Fig.31A**: CSE-tris; **Fig.31B**: choles-h-p; **Fig.31C**:2,2-Dim-TS.

Figure 32A-32D. Effect of indicated compounds on the growth of OCM1 ocular melanoma cancer cells. **Fig.32A**: TS-tris suspension (●) and TS-tris pellet (○); **Fig.32B**: Taxol (●), Taxol 50xTS-tris (○) and Taxol 50xTS-tris pellet (▼); **Fig.32C**: Taxol-S (●), Taxol-S with 50xTS-tris (○) and Taxol-S with 50xTS-tris pellet (▼); **Fig.32D**: Taxol-DS (●), Taxol-DS 50xTS-tris (○), and Taxol-DS 50xTS-tris pellet (▼).

Figure 33A-33E. Compounds representing new compositions of matter. **33A**: 2,2-Dim-TG; **33B**: 2,2-Dim-TG-tris; **33C**: T-DMAE-ether; **33D**: T-DMAE-ether oxalate; **33E**: T-DMAE-Q-ether methiodide.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

The current studies were undertaken in order to investigate the anti-tumor potential of several derivatives of Vitamin E, cholesterol, taxol and betulinic acid. In the Examples which follow, we demonstrate that the compounds of the present invention possess potent anti-tumor activity when tested against a wide variety of human cancer types. The compounds were tested against cancerous cell lines derived from liver, lung, colon, prostate, breast, cutaneous melanoma, ocular melanoma and leukemia. Though many compounds display similar antitumor activity in most of the human tumor cell lines tested, there are several exceptions.

For instance, most of the derivatives tested were less potent in killing hormone-dependent breast tumor cell line MCF-7 as compared with other tumor cell types. In addition, the methyl derivatives of TS (αT-MS and 2,2-Dim-TS) were especially active cytotoxic agents in the human lung tumor cell line, NIH-H-69. Furthermore, in contrast to most tumor cell types, human leukemia cells (HL-60) seemed insensitive to the toxic effects of tocopherol ester amines (e.g. T-TDAB-T). Interestingly, the Tris salts of TSE appeared dramatically more potent than TSE (free acid) with respect to cytotoxic action against tumor cells derived from the colon and liver. In contrast, the tris salt of TSE did not improve the antitumor activity of

TSE towards human melanoma cells. The succinate ester of betulinic acid was a potent antitumor agent for cutaneous melanomas but not for other tumor cell types. Our findings suggest that the active antitumor agents studied in these experiments are probably active against most tumor cell lines. Overall, the tocopherol amine containing compounds (e.g. T-TDAB-T, T-TDAB-Q, and T-TDAE-ether), the PEG ester compounds, the taxol succinate Tris compounds and choles-h-p were the most potent compounds in terms of cytotoxic abilities toward human tumor cell lines derived from liver, lung, colon, prostate, breast, cutaneous melanoma, ocular melanoma and leukemia. However, there is also some basis for selectivity in that certain compounds may prove advantageous with respect to the treatment of certain types of cancer. For each tumor cell line, specific examples of compounds that are the most effective in inhibiting specific tumor cell growth are summarized in each Example.

It is of interest to note that by replacing the succinic acid (a 4 carbon straight chain dicarboxylic acid) group on CS with phthalic acid (a 1,2 benzene dicarboxylic acid) the resulting compound, cholesterol hydrogen phthalate, is a more potent cytotoxic agent against all of the tumor types tested. Thus, we conclude that the attachment of a ring structure (benzene) that contains free carboxylic acids to the cholesterol molecule results in a compound that has a free carboxylic acid and has potent antitumor activity. Based on our previous studies with CS and its ether derivative CSE (which shows antitumor activity identical to CS), we predict that the attachment of a monocarboxylic acid benzene molecule to cholesterol through an ether linkage would also result in a cholesterol phthalate ether compound that would have potent antitumor activity, as well as be non-hydrolyzable, which is important for oral absorption. It is also predicted from our antitumor data with TS and TSE (the ether form of TS), which showed that these compounds have antitumor activity similar to CS and CSE, that phthalate esters and ethers of tocopherols, tocotrienols, taxols, and betulinic acids will also have excellent antitumor activity. Similarly, since our antitumor data with tocopherol amines (e.g. T-DMAB-T and T-DMAB-Q) show that when the carboxylic acid on the 4 carbon side chain of TS is replaced with a tertiary or quaternary amine, an improvement in terms of antitumor activity is observed. These data suggest that the replacement of the carboxylic acid on phthalate esters and ethers with an amine will also improve the antitumor activity of these compounds.

Vitamin E is a generic term that includes, in nature, eight substances, d-α-, d-β-, d-γ-, d-δ-tocopherol and d-α-, d-β-, d-γ-, d-δ-tocotrienol. Succinate esters for each of these substances can be made. We investigated whether the combination of several of these tocopherol and tocotrienol succinate esters would have antitumor activity. To accomplish this, a succinate ester of TRF was prepared (TRF-S) in the form of both a free acid and a tris salt. TRF-S is a mixture of vitamin E compounds containing the succinate ester of d-α- tocopherol (28%) and d-α- (35%), d-γ-(22%), and d-δ-tocotrienol (16%). The presence of the succinate ester of each of these vitamin E derivatives in the TRF-S preparation was confirmed by HPLC analysis using base hydrolysis. Our findings as shown in the examples below indicate that a mixture of tocopherol and tocotrienol esters (as, for example, TRF-S-tris or TRF-S free acid) are potent antitumor agents.

Implementation of the claimed invention will generally involve identifying patients suffering from tumors and administering the compounds in an acceptable form by an appropriate route. The dosage to be administered may vary, depending on the age, gender, weight and overall health status of the individual patient, as well as the nature of the cancer itself. The exact dosage will thus be determined on a case by case basis by the attending physician or other appropriate professional, but will generally be in the range of 1 to 100

mg/kg of body weight. Alternatively, the compound may be administered prophylactically to patients identified as being at risk for the development of cancer, either due, for example, to a genetic predisposition or as a result of exposure to a carcinogenic substance.

In a preferred embodiment of the invention, the compounds may be administered by injection either intravenously or parenterally. Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispensing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol.

Alternatively, the compounds may be administered by inhalation of an aerosol. This method has the advantage of delivering the antitumor compound directly to the lungs where it could, for example, provide protection against carcinogens such as those found in cigarette smoke and atmospheric pollutants, or effectively kill cancer cells located at this site. Those skilled in the art will recognize that a variety of inhalers appropriate for the practice of the invention are available, including those with various dose metering chambers, various plastic actuators and mouthpieces, and various aerosol holding chambers (e.g. spacer and reservoir devices) so that an appropriate dose of the Vitamin E compound can be delivered. Also, several non-ozone depleting (non-chlorofluorocarbon) propellants, such as various hydrofluoroalkanes (e.g. HFA 134a and HFA 227) are available. For administration via inhalation, the dose may be less than for other methods and will vary according to the exact delivery technology that is employed.

Administration may also be achieved transdermally using a patch impregnated with the compound, by ocular administration (eye drops), sublingual administration, nasal spray administration and rectal administration (suppository).

Administration may also be oral. In the case of oral administration, absorption of those compounds which are susceptible to inactivation by digestive enzymes may be accomplished by coating the compounds with an impermeable polymer membrane that is not susceptible to the action of digestive enzymes (duodenal esterases) or is biodegraded very slowly. In addition, amino acid polymers such as polylysine could be used. Impermeable polymer films would be degraded by microflora found in the colon. Thus, the compound would be released in a part of the intestine devoid of secreted digestive enzymes. However, it will be readily understood by those of skill in the art that other methods for preventing the hydrolysis of the compounds and promoting their absorption following oral administration can also be used in the practice of the present invention. For oral administration, the compounds may be administered in any of several forms, including tablets, pills, powders, lozenges, sachets, elixirs, suspensions, emulsions, solutions, syrups, aerosol, soft or hard gelatin capsules, or sterile packaged powders.

The compounds administered in according to the methods of the present invention may be administered as a composition which also includes a pharmaceutically acceptable carrier. The compounds may be mixed with a carrier, or diluted by a carrier, or enclosed within a carrier. When the carrier is a diluent, it may be a solid, semisolid or liquid material which acts as a vehicle, excipient or medium for the compound. Some examples of suitable carriers, excipients and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphates, alginate, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water syrup, methyl cellulose, methyl and propylhydroxybenzoates, talc, magnesium stearate and mineral oil. The formulations can also include lubricating agents, wetting agents, emulsifying agents, preservatives, and sweetening

or flavoring agents.

The compounds can be administered in the pure form or in a pharmaceutically acceptable formulation including suitable elixirs, binders, and the like or as pharmaceutically acceptable salts or other derivatives. It should be understood that the pharmaceutically acceptable formulations and salts include liquid and solid materials conventionally utilized to prepare injectable dosage forms and solid dosage forms such as tablets and capsules. Water may be used for the preparation of injectable compositions which may also include conventional buffers and agents to render the injectable composition isotonic. Solid diluents and excipients include lactose, starch, coatings and the like. Preservatives such as methyl paraben or benzalkium chloride may also be used. Depending on the formulation, it is expected that the active composition will consist of 1-99% of the composition and the vehicular "carrier" will constitute 1-99% of the composition.

In particular, one advantage of the present invention involves the oral administration of TSE, 2,2-Dim-TS, 2,2-Dim-TG and T-DMAE ether. Previous to the practice of the present invention, it was not possible to effectively administer known derivatives of Vitamin E orally due to the fact that they were enzymatically hydrolyzed by esterases *in vivo*. In contrast, based on their structure, the derivatives TSE, 2.2-Dim-TS, 2,2-Dim-TG and T-DMAE ether are non-hydrolyzable and thus high levels of bioavailability are maintained until they are taken up by cells (see Example 9).

One aspect of the present invention contemplates administering to a patient a compound by the methods of the present invention in combination with administering to the patient an acceptable chemotherapeutic agent or combination of agents. As used herein "chemotherapeutic agent" means any chemical agent or drug used in chemotherapy treatment which selectively affects tumor cells, including but not limited to such agents as taxanes (e.g. taxol), adriamycin, amscrine, etoposide, cisplatinum, vincristine, vinblastine and methotrexate. Other such agents are well-known in the art. It is anticipated that the compounds of the present invention may be administered either in combination with or separately from the chemotherapeutic agent(s). The dose will vary depending on a variety of factors including the route of administration, choice of chemotherapeutic agent, etc.

Another aspect of the present invention is to administer compounds by the methods of the present invention in combination with treating the patient by exposing the patient to ionizing radiation. The protocols for traditional radiation therapy, e.g. γ-radiation. are known, readily available, and routinely practiced by those of skill in the art. These include established protocols for the administration of drugs in combination with radiation therapy [Wobst et al. (1998) *Ann.Oncol.* 9, 951-962]. The dose of ionizing radiation will vary depending on a variety of factors including intensity, source of radiation, etc.

Another aspect of the present invention is the administration of compounds by the methods of the present invention in order to prevent metastasis of cancer. For example, the compounds may be administered following the surgical removal of a tumor. The compound may also be administered prophylactically to patients identified as being at risk for the development of cancer.

The compounds which are administered in the practice of the present invention may be administered singly (i.e. only one derivative compound administered at a time) or in combination (i.e. more than one derivative compound administered at a time).

Numerous studies have shown that the compounds utilized in the practice of the present invention appear to be safe when administered *in vivo*. In fact, numerous studies clearly demonstrate that the *in vivo* and *in vitro* administration of tris salts and PEG esters of

TS and other vitamin E and cholesterol derivatives protect tissue, cells and subcellular fractions from toxic insults (8, 9,11, 13, 14). Fariss et al. (6) have shown that the growth of normal bone marrow cells (cells *in vivo* that are usually sensitive to the toxic effects of cancer chemotherapeutic drugs) isolated from mice are not inhibited by treatment with TS, CS, TSE and CSE at concentrations up to 200μM. Other investigators have also shown that TS administration does not adversely affect normal cells (2, 3, 13) in contrast to its effect on tumor cells. Furthermore, after the administration of the antitumor agents TS-T, TS-PEG, and TSE-T to rats at a single dose of 0.19 mmol/kg (approx. 100 mg/kg), ip, the tissue histopathology (liver, lung, heart, brain and kidney) was normal 24 hours following administration.

In another aspect, the present invention provides five novel compounds(see Figure 33), namely

1) d-α-tocopheryl 2,2-dimethylglutarate
2) d-α-tocopheryl 2,2 dimethyglutarate TRIS salt
3) d-α-tocopheryl β-(N,N-dimethylamino)ethyl ether (free base)
4) d-α-tocopheryl β-(N,N-dimethylamino)ethyl ether oxalate salt, and
5) d-α-tocopheryl β-N,N-(dimethylamino)ethyl ether methiodide.

The compounds may be used in the practice of the present invention as antitumor agents. Details of the syntheses of the five new compounds are given in Examples 10-14 below. Those of skill in the art will recognize that certain modifications of these compounds may be made in order to, for example, optimize bioavailability, solubility, potency, and the like, or for any other reason. All such modifications of these compounds and the derivative compounds which result from such modifications are intended to be encompassed by the present application.

The following examples illustrate the use of the derivatives of Vitamin E, cholesterol, taxol and betulinic acid as antitumor agents. The details of the examples should not be construed to unduly limit this invention.

EXAMPLES

Materials

The human cells lines used in the present investigation and their origins are as follows: Hep 3B (liver); NIH-H69 (lung); HT29 (colon); Du145 (prostate); MCF7 and MD-231 (breast); A375 (cutaneous melanoma); OCM1 (ocular melanoma); HL60 (leukemia). All cell types except OCM1 are available from the American Type Culture Collection (ATCC). OCM1 cells were obtained from Dr. June Kan-Mitchell at the U.of California, San Diego.

The anti-tumor agents of the present invention, except as noted, were synthesized by Dr. J. Doyle Smith, Virginia Commonwealth Univ., Richmond, VA. The succinate esters, PEG esters, butyrate ethers and the tris salts were prepared by procedures similar to those described in U.S. Patent 5,610,180 to Fariss.

T-DMAB-T was prepared as follows: In a flask equipped with a magnetic stirrer and reflux condenser was placed 553mg (3.3mM) of γ-(dimethylamino)butyric acid and 2 ml (18.9 mM) of $POCl_3$. The mixture was stirred and heated at 93°C for 1 hour to give a clear,

colorless solution. The excess $POCl_3$ was removed under reduced pressure with warming to give an oil, which is the crude γ-(dimethylamino)butyryl chloride. To this was added 1.02g of d-α-tocopherol and 3 ml of $CHCl_3$. The mixture was stirred and warmed to 53°C for 4 days. This mixture was cooled and then treated with 3g of ice, 6ml of 5% Na_2CO_3 solution, and 6ml of $CHCl_3$ to form an emulsion. After cooling in the refrigerator overnight, the layers separated and the $CHCl_3$ was removed. The water layer was again extracted several times with $CHCl_3$ and the combined extracts were dried over $MgSO_4$. This extract, which contained the crude product, was chromatographed using a column of silica gel and $CHCl_3$/methanol. The desired product was isolated as a pale, yellow liquid; 968 mg (75%). IR (thin film) Absorption at 1757 cm^{-1} shows the presence of an ester. TLC ($CHCl_3$/methanol, 9:1) Rf = 0.54; single spot.

T-DMAB-Q was prepared as follows: to a cooled vial containing 336mg (0.618mM) of d-α-tocopheryl γ-(dimethylamino) butyrate was added 1.5g (a large excess) of methyl iodide and 1ml of $CHCl_3$. The vial was stoppered and the reaction was allowed to proceed at room temperature for 24 hours. Reduced pressure was used to remove the solvents and form the crude product; 345mg (81%). After multiple recrystallizations using acetone/ethyl acetate, tan crystals were obtained; 222mg (52%); mp 165-167°C. Anal ($C_{36}H_{64}NO_3I$) CHN. TLC ($CHCl_3$/methanol, 9:1), Rf = 0.22, single spot.

The exceptions are as follows: α-TS, PEG 1000, choles-h-p and CS-tris were purchased from Sigma Chem. Co. and T-P-Na_2 was purchased from U.S. Biochemical Co.; α-T, γ-T and δ-T were gifts from Henkel Corp.; TS-PEG, Rice-B-oil fraction, Palm oil fraction, γ-T3, and γ-T3 acetate were gifts from Dr. John Hyatt, Eastman Chemical Co.; TRF was a gift from Dr. Paul Sylvester (Washington State Univ.) and was originally obtained from the Palm Oil Research Institute of Malaysia; betulinic acid was a gift from Dr. Joun Pezzuto, University of Illinois, Chicago. Other antitumor compounds were synthesized by Dr. J. Doyle Smith, Virginia Commonwealth University, Richmond, VA. The succinate esters, PEG esters, butyrate ethers and the tris salts were prepared by procedures previously described (US Patent No.).

All test compounds (except tris salts and betulinic acid) were dissolved in 100% ethanol and administered to cells such that the final concentration of ethanol in medium was less the 0.2%. Tris salt derivatives were administered in distilled water, following two 15 second sonications. Betulinic acid was administered in dimethylsulfoxide (DMSO).

Method for anti-tumor compound screening

Alamar Blue Assay:

Alamar blue dye was used to evaluate cell survival and proliferation. Living cells metabolize the non-fluorescent dye to a fluorescent metabolite which can be detected by a fluorescence plate reader. There is a positive correlation between the level of fluorescence and the number of living cells. The fluorescence intensity of the cells treated with a test compound was compared to that of a control group which has no added test compound (vehicle only). The result was expressed as "Cell number (% control)". A reduction in the cell number indicates inhibition of cell growth, or an increase in cell death.

Procedure:

1) On day one, cells were plated at a density of 2.5 or 5 x 10^3 cells/well, depending on the cell type, in a 96-well flat-bottomed plate in RPMI 1640 with 10% fetal bovine serum for the following cell lines: HL-60, NIH-H69 and HT29; and in DMEM with 10% fetal bovine serum

serum for all other cell lines. On the second day, the medium was replaced with 200μL RPMI 1640 (10% fetal bovine serum) medium containing the desired concentration of test compound. The concentration of test compound used ranged from 0 to 50μM. 200μL of medium without cells was plated as a blank.

2) Cells were maintained in a humidified atmosphere in 5% CO_2 at 37°C for 42-70 hours, depending on the cell type. 2-6 hours prior to the end of exposure to the test compound, 10-20μL of Alamar blue stock solution was added to each well. After incubation at 37°C for an additional 2-6 hours, the plate was maintained at room temperature for 30 minutes. The exact time of addition of Alamar blue, the precise amount of Alamar blue added, and the length of incubation with Alamar blue varied depending on the cell type and level of activity of the compound being tested. After 30 minutes at room temperature, the fluorescence intensity of each well was measured using a CytoFluor series 4000 multiplate reader (excitation wavelength, 530nm; emission, 590nm; gain. 40). Fluroescent values from blank cells were subtracted from fluorescent values of cells treated with test compounds. The resulting values were then divided by the corresponding values obtained from the control samples to give the number of viable cells (% control). The IC_{50} values (concentration required to inhibit cell growth by 50%) were extrapolated from the plot of cell number (%control) versus compound concentration. The cell number (% control) plotted was the mean ± SD (n=6).

Animals

Male Sprague-Dawley rats from Simonsen Labs (Gilroy, CA) weighing 175-225 g were used throughout the course of this study. Animals received water and food (Purina Rat Chow 5001, Ralston Purina, St. Louis, MO) *ad libitum* for at least three days prior to the onset of the experiment. Powdered TS-tris, TSE-tris and TS-PEG were suspended in saline with brief sonication (30 sec) and were given intraperitoneally at a dose of 0.19 mmol/kg body weight. Saline was given to rats at a dose of 4 ml/kg. received CCl_4 by oral lavage 6 or 18 h after tocopherol administration.

After 24 hours, rats were anesthetized with diethyl ether, and blood samples (4-5 ml) were withdrawn from the inferior aorta. Blood samples were immediately mixed with 15 mg tripotassium EDTA, and aliquots were centrifuged at low speed to prepare plasma samples. All procedures were approved by the Washington State University Animal Care and Use Committee.

Tocopherol determinations

Tocopherol and tocopherol ester levels were measured according to the methods described by Fariss et al. (11). TSE levels were measured according to the procedures of Tirmenstein et al. (12). Samples were analyzed by reversed-phase high-performance liquid chromatography equipped with fluorimetric detection. Retention times for *d*-δ-tocopherol (internal standard), α-T and TSE were 8.0, 11.5 and 13.4 min respectively.

Preparation of Taxol and TS-tris Suspensions and Liposomes:

TS-tris suspensions were prepared by adding 1 ml of water or saline to 30 mg of TS-tris in a microfuge tube and sonicating for 15 sec., twice. For taxol-TS-tris suspensions, 1 mg of taxol or taxol monosuccinate tris salt or taxol disuccinate tris salt) was added to 30 mg of TS-tris prior to the addition of water or saline and sonication. (Therefore on a molar basis there is 50 times more TS-tris than taxol). To prepare washed liposomal suspensions, the

suspensions mentioned above were spun at 10,000rpm for 15 min and the resulting pellet (liposomes) was washed with saline or water. This procedure (spinning and washing) was conducted 3 times and the final washed liposomal pellet was resuspended by sonication in the original volume of water or saline. The presence of liposomes in each suspension was confirmed by light microscopy.

EXAMPLE 1.

Testing of compounds in human liver tumor (Hep3B) cells

2.5×10^3 cells/well were plated in a 96-well flat bottom plate. On the next day, the medium was replaced with 200μL RPMI 1640 (10% FBS) containing from 0 to 50μM of test compound. Cells were maintained as described in Methods. After 70 hours, 10μL of Alamar blue stock solution was added to each well and the plates were incubated at 37°C for an additional 2 hours. Plates were then kept at room temperature for 30 min., following which fluorescence intensity was measured and the values obtained were processed as described in Methods. The results are given in Figure1A-F, Figure 2A-F, Figure 3A-F, Figure 4A-F, Figure 5A-F and figure 6, and Table 1 gives the corresponding IC_{50} values. As can be seen, the compounds that appear to be most effective in inhibiting the growth of human Hep 3B cells are T-DMAB-T, T-DMAB-Q, T-DMAE-ether, TS-PEG, TSE-PEG, TRF-S, TRF-S-T, γ-T3, Choles-h-p, and Btl-acid. Antitumor activity was also observed for all of the compounds tested except α-T, γ-T, T-P-Na_2, PEG1000, and γ-T3 acetate.

Table 1. IC_{50} (μM) of Test Compounds in Human Liver Cancer (Hep 3B) Cells

Compound	IC_{50} (μM)
αTS	27
αTS-T	22
αT-MS	28
TSE	50
TSE-T	35
δTS	37
γTS-T	47
2,2-Dim-TS	26
T-DMAB-T	6
T-DMAB-Q	19, 12
δT	45
γT	>50
T-P-Na_2	>50

PEG 1000	>50
TS-PEG	8
TSE-PEG	16
TRF	26
TRF-S	17
TRF-S-T	16
Rice B-oil	76
Palm oil	87
γT3	25
γT3-S	45
γT3-acetate	>50
CS-tris	24
CS-PEG	31
CSE-tris	32
Choles-h-p	13
Btl-acid	15
Btl acid-S	~50
T-DMAE ether	14

EXAMPLE 2.

Testing of Compounds in Human Prostate Cancer (Du145) Cells

2.5×10^3 cells/well were plated in a 96-well flat bottom plate. On the next day, the medium was replaced with 200μL RPMI 1640 (10% FBS) containing from 0 to 50μM of test compound. Cells were maintained as described in Methods. After 70 hours, 10μL of Alamar blue stock solution was added to each well and the plates were incubated at 37°C for an additional 2 hours. Plates were then kept at room temperature for 30 min., following which fluorescence intensity was measured and the values obtained were processed as described in Methods. The results are given in Figure 7A-F, Figure 8A-F, and Figure 9A-D, and Table 2 gives the corresponding IC_{50} values. As can be seen, the compounds that appear to be most effective in inhibiting the growth of human prostate cancer (Du 145) cells are T-DMAB-T, T-DMAB-Q, α-TS-T, TS-PEG, TSE-PEG, γ-T3 and Choles-h-p. Antitumor activity was also observed for all of the compounds tested except PEG1000.

Table 2. IC_{50} (μM) of Test Comp unds in Human Prostate Cancer (Du 145) Cells

C mpound	IC_{50} (μM)
αTS	34
αTS-T	23
αT-MS	31
TSE	>50
2,2-Dim-TG	37
2,2-Dim-TS	30
T-DMAB-Q	21
T-DMAB-T	10
PEG 1000	>50
TS-PEG	15
TSE-PEG	22
TRF	42
TRF-S	32
γT3	24
γT3-S	36
CS-tris	~50
CS-PEG	32
Choles-h-p	11

EXAMPLE 3.

Testing of compounds in human small cell lung cancer (NIH-H69) cells

5×10^3 cells/well were plated in a 96-well flat bottom plate. On the next day, the medium was replaced with 200μL RPMI 1640 (10% FBS) containing from 0 to 50μM of test compound. Cells were maintained as described in Methods. After 66 hours, 20μL of Alamar blue stock solution was added to each well and the plates were incubated at 37°C for an additional 6 hours. Plates were then kept at room temperature for 30 min., following which fluorescence intensity was measured and the values obtained were processed as described in Methods. The results are given in Figure 10A-F, Figure 11A-F, Figure 12A-E, Figure 13A-F, and Figure 14A-E, and Table 3 gives the corresponding IC_{50} values. As can be seen, the compounds that appear to be most effective in inhibiting the growth of human small cell lung cancer (NIH-H69) cells are α-T-MS, T-DMAB-Q, T-DMAB-T, TS-PEG, TSE-PEG, TRF-S, γ-T3, choles-h-p, 2,2-Dim-TS, and CS-tris. Antitumor activity was observed for all

compounds tested except γ-T, δ-T, PEG 1000, γ-T3-acetate and T-P-Na_2.

Table 3. IC_{50} (μM) of Test Compounds in Human Lung Cancer (NIH-H69) Cells

Compound	IC_{50} (μM)
αTS	33
αTS-T	40
αT-MS	18
TSE	24, 46
δTS	~50
γTS-T	~50
2,2-Dim-TS	11
T-DMAB-Q	13
T-DMAB-T	8
δT	>50
γT	>50
T-P-Na_2	>50
PEG 1000	>50
TS-PEG	8
TSE-PEG	15
TRF	37
TRF-S	25
TRF-S-T	30
Rice B-oil	>50
Palm oil	99
γT3	21
γT3-S	37
γT3 acetate	>50
CS-tris	20
CS-PEG	25

CSE-tris	33
Choles-h-p	8

EXAMPLE 4.

Testing of compounds in human colon cancer (HT29) cells

2.5×10^3 cells/well were plated in a 96-well flat bottom plate. On the next day, the medium was replaced with 200μL RPMI 1640 (10% FBS) containing from 0 to 50μM of test compound. Cells were maintained as described in Methods. After 70 hours, 10μL of Alamar blue stock solution was added to each well and the plates were incubated at 37°C for an additional 2 hours. Plates were then kept at room temperature for 30 min., following which fluorescence intensity was measured and the values obtained were processed as described in Methods. The results are given in Figure 15A-F, Figures 16A-E, Figure 17A-F, Figure 18A-B, and Table 4 gives the corresponding IC_{50} values. As can be seen, the compounds that appear to be most effective in inhibiting the growth of human colon cancer (HT29) cells are α-TS-T, T-DMAB-Q, T-DMAB-T, TS-PEG, TRF-S, and choles-h-p. Antitumor activity was also observed for all of the compounds tested except TSE and PEG1000.

Table 4. IC_{50} (μM) of Test Compounds in Human Colon Cancer (HT29) Cells

Compound	IC_{50} (μM)
α-TS	22
α-TS-T	18
αT-MS	24
TSE	>50
TSE-T	25
2,2-Dim-TG	28
2,2-Dim-TS	29
T-DMAB-Q	19
T-DMAB-T	18
PEG 1000	>5
TS-PEG	14
TSE-PEG	24
TRF	32
TRF-S	14
γT3	30

γT3-S	26
CS-tris	24
CS-PEG	31
CSE-tris	24, >50
Choles-h-p	11

EXAMPLE 5.

Testing of compounds in human breast cancer (MCF-7 and MD-231) cells

5 x 10^3 cells/well were plated in a 96-well flat bottom plate. On the next day, the medium was replaced with 200μL RPMI 1640 (10% FBS) containing from 0 to 50μM of test compound. Cells were maintained as described in Methods. After 68 hours, 10μL of Alamar blue stock solution was added to each well and the plates were incubated at 37°C for an additional 4 hours. Plates were then kept at room temperature for 30 min., following which fluorescence intensity was measured and the values obtained were processed as described in Methods. The results for MCF-7 cells are given in Figure 19A-E and Figure 20A-C; the results for MD-231 cells are given in Figure 21A-D and Figure 22A-D; and Table 5 gives the corresponding IC_{50} values.

As can be seen, the compounds that appear to be most effective in inhibiting the growth of hormone- dependent human breast cancer (MCF7) cells is TS-PEG. Antitumor activity was, however, also observed for all of the compounds tested except PEG 1000.

The most effective compounds for inhibiting the growth of hormone-independent MD-231 human breast cells were TS-PEG, TRF-S, and DMAB-T.

Table 5. IC_{50} (μM) of Test Compounds in Hormone-Dependent (MCF-7) and Hormone-Independent (MD-231) Human Breast Cancer Cells

Compound	MCF-7 Cells	MD-231 Cells
	IC_{50} (μM)	IC_{50} (μM)
αTS	41	25
αT-MS	42	
TSE	~50	50
2,2-Dim-TG	42	
T-DMAB-T		18
PEG 1000	>50	
TS-PEG	15	10
TSE-PEG	36	
TRF	42	38

TRF-S	37	22
γT3	27	
γT3-S	41	
CS-tris	~50	
CS-PEG	45	
α-TS-tris		28
2,2-Dim-TS		28

EXAMPLE 6.

Testing of compounds in human leukemia cancer (HL60) cells

5 x 10^3 cells/well were plated in a 96-well flat bottom plate. On the next day, the medium was replaced with 200μL RPMI 1640 (10% FBS) containing from 0 to 100μM of test compound. Cells were maintained as described in Methods. After 42 hours. 20μL of Alamar blue stock solution was added to each well and the plates were incubated at 37°C for an additional 6 hours. Plates were then kept at room temperature for 30 min., following which fluorescence intensity was measured and the values obtained were processed as described in Methods. The results are given in Figure 23A-D, and Table 6 gives the corresponding IC_{50} values. As can be seen, the compounds that appear to be most effective in inhibiting the growth of human leukemia (HL-60) cells are TS-PEG, Choles-h-p and γ-T3. Antitumor activity was also observed for all of the compounds tested except γ-T, PEG 1000, and CS-PEG.

Table 6. IC_{50} (μM) of Test Compounds in Human Leukemia (HL-60) Cells

Compound	IC_{50} (μM)
αTS	27
αT-MS	24
TSE	>50
δTS	27
γTS-T	34
δT	46
γT	>50
T-DMAB-T	39
2,2-Dim-TS	24
PEG 1000	>50

TS-PEG	12
TSE-PEG	24
TRF	39
TRF-S	40
TRF-S-T	40
γT3	15
γT3-S	36
γT3-S-tris	36
γT3-acetate	>50
CS-tris	27
CS-PEG	>50
Choles-h-p	13

EXAMPLE 7.

Testing of compounds in human cutaneous melanoma cancer (A375) cells

2.5×10^3 cells/well were plated in a 96-well flat bottom plate. On the next day, the medium was replaced with 200μL RPMI 1640 (10% FBS) containing from 0 to 50μM of test compound. Cells were maintained as described in Methods. After 70 hours, 10μL of Alamar blue stock solution was added to each well and the plates were incubated at 37°C for an additional 2 hours. Plates were then kept at room temperature for 30 min., following which fluorescence intensity was measured and the values obtained were processed as described in Methods. The results are given in Figure 24A-D, Figure 25A-C, and Figure 26A-D, and Table 7 gives the corresponding IC_{50} values. As can be seen, the compounds that appear to be most effective in inhibiting the growth of human cutaneous melanoma (A375) cells are TS, TS-T, αT-MS, TS-PEG. TSE-PEG, TRF-S, Btl-acid, Btl-acid-S, T-DMAB-T, and γ-T3. Antitumor activity was also observed for all of the compounds tested.

Table 7. IC_{50} (μM) of Test Compounds in Human Cutaneous Melanoma (A375) Cells

Compound	IC_{50} (μM)
αTS	25
αT-MS	27
TSE	43
TS-PEG	15
TSE-PEG	29
TRF	34

TRF-S	26
γT3	17
γT3-S	32
CS-tris	32
CS-PEG	29
Btl-acid	14
Btl-acid-S	25
2,2-Dim-TS	33
T-DMAB-T	23
TS-tris	26
TSE-tris	>50

EXAMPLE 8.

Testing of compounds in human ocular melanoma cancer (OCM1) cells

2.5×10^3 cells/well were plated in a 96-well flat bottom plate. On the next day, the medium was replaced with 200μL RPMI 1640 (10% FBS) containing from 0 to 50μM of test compound. Cells were maintained as described in Methods. After 70 hours, 10μL of Alamar blue stock solution was added to each well and the plates were incubated at 37°C for an additional 2 hours. Plates were then kept at room temperature for 30 min., following which fluorescence intensity was measured and the values obtained were processed as described in Methods. The results are given in Figure 27A-E. Figure 28A-F, Figure 29A-F, Figure 30A-D, and Figure 31A-C, and Table 8 gives the corresponding IC_{50} values. As can be seen, the compounds that appear to be most effective in inhibiting the growth of human ocular melanoma (OCM-1) cells are choles-h-p, Btl-acid, TS-PEG, T-DMAB-Q, and T-DMAB-T.

Experiments were conducted to determine if taxol, which is insoluble in water: (a) could be solubilized by adding this compound to TS-tris followed by the addition of water and sonication (which produces liposomes); (b) could retain its antitumor activity if solubilized in a suspension of TS-tris; (c) would be sequestered by the TS-liposomes, thus conferring excellent antitumor activity on the TS-tris liposomes that are isolated and washed from the TS-tris/taxol suspension; and (d) could upon esterification with succinate and tris salt formation (to produce the taxol monosuccinate tris salt and the taxol disuccinate tris salt), have greater solubility in water, retain its antitumor activity and be sequestered in TS-tris liposomes. The data illustrated in Table 8 and Figure 32A-D clearly demonstrates that the answer to all of these questions is yes.

In regards to the solubilization of taxol by TS-tris, we observed using light microscopy that taxol in water formed a precipitate that had a distinct appearance upon magification under the microscope and could be pelleted by centrifugation. However when taxol was combined with TS-tris, the resulting suspension formed liposomes without the precipitate, again observed using the light microscope. As can be seen in Fig 32B, the suspension of taxol plus TS-tris (referred to as taxol, 50X TS-tris) and the washed liposomes obtained from this

suspension (referred to as taxol,50X TS-tris pellet) had excellent antitumor activity [killing greater than 85% of the tumor cells at 10 nM taxol (also containing 0.5 micromolar TS, a nontoxic dose as seen in Fig 32A)]. In fact the antitumor activity of these two preparations was nearly identical to that of taxol dissolved inethanol (referred to as Taxol). These results indicate that taxol can be solubilized by TS-tris in water or saline, that taxol is contained within TS-tris liposomes (probably as part of the lipid layer) and that both the TS-tris/taxol suspension and liposomal fractions have excellent antitumor activity. In addition, our data suggest that the addition of TS-tris and taxol allows for 100% kill of tumor cells that normally is not observed with taxol alone (see Fig.32B). These findings suggest that both taxol and TS-tris are killing tumor cells by different mechanisms and that the administration of both of these compounds in one preparation may improve the antitumor activity of both compounds.

The data with taxol monosuccinate tris salt treatment in combination with TS-tris suspension and liposomes also demonstrates the same increased solubility for this taxol derivative in the presence of TS-tris as that observed for taxol. Like taxol, the monosuccinate tris of taxol has minimal solubilty in water (as determined by centrifugation and light microscopy). In contrast the disuccinate tris salt of taxol displayed complete solubility in water at a concentration of 4 mg/ml. These data suggest that the disuccinate taxol may serve to provide the means to administer taxol parenterally. Unfortunately, the antitumor activity of the disuccinate taxol is not as great as taxol and the monosuccinate derivative. However, once *in vivo,* the hydroylsis of this compound by esterases could liberate taxol and monosuccinate taxol which are much more active. The monosuccinate tris salt of taxol demonstrates excellent antitumor activity both in ethanol and as a suspension in TS-tris. The loss of antitumor activity of the liposomal preparation suggests a greater water solubility for this derivative. In addition, our data suggest that the addition of TS-tris and monosuccinate and disuccinate taxol allows for 100% kill of tumor cells that normally is not observed with taxol derivative alone (see Fig.32 C and 32D). These findings suggest that these taxol derivatives and TS-tris are killing tumor cells by different mechanisms and that the administration of both of these compounds in one preparation may improve the antitumor activity of both compounds.

Table 8. IC_{50} (μM) of Test Compounds in Human Ocular Melanoma (OCM1) Cells

Compound	IC_{50} (μM)
αTS	29
αTS-T	20
αT-MS	21
TSE	33
TSE-T	32
2,2-Dim-TG	35
2,2-Dim-TS	25, 22
T-DMAB-T	17, 21
T-DMAB-Q	14, 10

PEG 1000	>50
TS-PEG	14
TSE-PEG	22
TRF	42
TRF-S	28
γT3	26
γT3-S	34
CS-tris	23
CS-PEG	33
CSE-tris	29
Choles-h-p	14, 14
Btl-acid	15
Btl-acid-S	49
Taxol (in ETOH)	<0.01
Taxol-S tris (in ETOH)	<0.01
Taxol-DS tris (in ETOH)	>1.0
Taxol + TS-tris (sonicated in water)	<0.01
liposomes isolated from Taxol + TS-tris	<0.01
Taxol-S tris + TS-tris (sonicated in water)	<0.01
liposomes from Taxol-S tris + TS-tris	<0.1
Taxol-DS tris + TS-tris (sonicated in water)	>0.1
liposomes from Taxol-DS tris + TS-tris	>1.0

EXAMPLE 9.

In vivo plasma concentrations of tocopherol analogs 24 hours after administration

The plasma concentrations of tocopherol analogs were assessed *in vivo* in rats 24 hours after administration. Rats were given either an intrapeironeal (ip) injection or an oral intubation of TS-tris salt, TSE or TS-PEG (0.19mmol/kg body weight). After 24 hours, rats were sacrificed and blood samples were obtained and extracted for HPLC analysis of concentrations of TS-PEG, TS, TSE and T.

As can be seen in Table 9 below, the *in vivo* administration of TS-tris and TS-PEG result in plasma levels of these compounds that suggest they may be active anti-tumor agents *in vivo*. Furthermore, the near identical TSE plasma concentrations for rats administered

TSE-tris by ip and oral administration demonstrates the excellent oral absorption of this compound (a similar plasma level was also observed for the oral administration of TSE, data not shown). These data indicate that TSE (and other nonhydrolyzable vitamin E and cholesteral derivatives, such as the 2,2 dimethyl TS) may be given orally to treat cancer in patients.

Table 9. *In vivo* plasma concentrations of tocopherol analogs 24 hours after administration.

Analog Administered	Plasma concentration (nmol/ml)[a]			
	T	TS	TS-PEG	TSE
Vehicle (saline)	11.6 ± 1.1	nd[b]	nd	nd
TS-tris (ip)	24.4 ± 5.6	29.8 ± 7.2	nd	nd
TS-PEG (ip)	22.8 ± 0.8	38.4 ± 7.3	10.6 ± 3.0	nd
TSE-tris (ip)	18.7 ± 1.5	nd	nd	11.3 ± 2.5
TSE-tris (oral)	12.5 ± 2.2	nd	nd	9.2 ± 1.7

[a], values are the means ± SD (n=4)
[b], not determined

EXAMPLE 10. Synthesis of d-α-tocopheryl 2,2-dimethylglutarate (Figure 33A).

In a small reaction vial containing a magnetic stirring bar was placed 1.54 g (3.58 mM) of d-α-tocopherol, 569 mg (4.00 mM) of 2,2 dimethylglutaric anhydride, and 15 mg of anhydrous potassium carbonate. the vial was swept with nitrogen gas, capped, and placed in an oil bath at 140 °C for 8 days. the vial was opened every 2 days and an additional 120 mg of the 2,2-dimethylglutaric anhydride was added giving a total of 360 mg (2.5 mM) of the anhydride added during the heating. To the reaction mixture was added 15 ml of chloroform and 25 ml of water. This mixture was stirred at 38°C for 16 hr to hydrolyze the unreacted anhydride. The chloroform layer was removed and dried over anhydrous magnesium sulfate. The crude product was run through a silica column using hexane-chloroform, chloroform, and chloroform-methanol as the mobile phases. Several fractions were considered to be pure product; 768 mg (37%). A number of fractions were mixtures and were run through a second silica column to give an additional 252 mg (12 %) of pure product. The total yield was 1.02 g (49%). The compound is a low-melting solid. IR (thin film) showed that the compound absorbs at 1755 cm^{-1} (indicative of an ester) and at 1702 cm^{-1} (indicative of C00H). Thin layer chromatography (TLC) in chloroform-methanol 9:1 yielded an Rf value of 0.63.

EXAMPLE 11. Synthesis of d-α–tocopheryl 2,2 dimethyglutarate TRIS salt (Figure 33B).

In a small round bottom (R.B.) flask was placed 1.31 g (2.28 mM) of d-α-tocopheryl 2,2-dimethylglutarate in 10 ml of chloroform. In a separate container 263 mg (2.17 mM) of TRIS (base) was dissolved in 10 ml of methanol. The methanol solution was poured into the R.B. flask to give a clear solution. The solvents were removed under reduced pressure to give an oil which solidified upon the addition of acetone. The material was recrystallized using

acetone to give a white solid, 1.42 g (90 %) with a melting point of 96-99 °C. IR (thin film) showed that the compound absorbs at 1744 cm^{-1} (ester), and at 1557 cm^{-1} indicative of a -COO^- salt, but no absorption at 1702 cm^{-1}, i.e no -COOH group is present.

Elemental analysis was conducted and the results were compared to the theoretical results which would be expected from a compound with the formula $C_{40}H_{71}NO_8$:

Theoretical:	C, 69.22	H, 10.31	N, 2.02
Actual Results:	C, 69.19	H, 10.36	N, 2.10

EXAMPLE 12. Synthesis of d-α-tocopheryl β-(N,N-dimethylamino)ethyl ether (free base) (Figure 33C).

In a small reaction vial with a magnetic stirring bar was placed 800mg (1.86 mM) of d-α-tocopherol, 445 mg (11.1 mM) of NaOH (crushed), and 2 ml of methyl ethyl ketone (MEK). After the mixture had stirred for 15 minutes, 535 mg (3.71 mM) of 2-dimethylaminoethyl chloride hydrochloride was added; the vial was swept with nitrogen gas; the vial was capped and the mixture heated for 4 hours at 78 °C. The liquid phase of the mixture was transferred to a R.B. flask and the remaining solid was extracted repeatedly with 4 ml portions of acetone. The acetone extracts were added to the initial liquid phase. The solvents were removed under reduced pressure to give a pale yellow oil. This oil was passed through a silica column using chloroform as the mobile phase to give 814 mg (87%) of the d-α-tocopheryl β-(N,N-dimethylamino) ethyl ether (free base). Thin layer chromatography (TLC) in chloroform-methanol 9:1 yielded an Rf value of 0.65.

H NMR ($CDCl_3$) δ 0.80-1.60(brm, 36H, CH_2),
1.77 (m, 2H, CH_2),
2.07-2.18 (s, 9H, three CH_3),
2.35 (s,6H, $N(CH_3)_2$),
2.56 (t, 2H, CH_2),
2.72 (t, 2H, CH_2),
3.75 (t, 2H, CH_2).

Elemental analysis was conducted and the results were compared to the theoretical results which would be expected from a compound with the formula $C_{33}H_{59}NO_2$

Theoretical:	C 78.98	H 11.85	N 2.79
Actual results:	C 78.92	H 12.06	N 2.83

EXAMPLE 13. Synthesis of d-α-tocopheryl β-(N,N-dimethylamino)ethyl ether oxalate salt (Figure 33D).

In one container was placed 238 mg (0.47 mM) of d-α-tocopheryl β-(N,N-dimethylamino)ethyl ether (free base) in 4 ml of acetone. In a separate container 60.0 mg (0.47 mM) of oxalic acid dihydrate was dissolved in 4 ml of acetone. This oxalic acid solution was added to the amine (free base) solution to give a large amount of white solid. This was filtered off and recrystallized from acetone to give 237 mg (85%) of a white solid with a melting point of 170-172 °C. Infrared (KBr) studies showed that the compound absorbs at 2523 cm^{-1} (indicative of a tertiary amine salt) and at 1650-1580 cm^{-1} (indicative of a -COO^- salt).

Elemental analysis was conducted and the results were compared to the theoretical

results which would be expected from a compound with the formula $C_{35}H_{61}NO_6$.

Theoretical:	C 71.02	H 10.39	N 2.37
Actual results:	C 70.97	H 10.45	N 2.49

EXAMPLE 14. Synthesis of d-α-tocopheryl β-N,N-(dimethylamino)ethyl ether methiodide (Figure 33E).

In a small reaction vial with a magnetic stirring bar was placed 76 mg (0. 151 mM) of d-αtocopheryl β-(N,N-dimethylamino) ethyl ether (free base). The vial was cooled and 1.20 g (8.45 mM) of methyl iodide was added; the vial was capped and the mixture allowed to stir at R.T. for 3 days. The vial was opened and warmed to remove the excess methyl iodide forming a solid. This solid was recrystallized several times using acetone then dried over phosphorous pentoxide (under vacuum) to give 95 mg (97%) of white crystals with a melting point of 178-180 °C. Thin layer chromatography (TLC) in chloroform-methanol 9:1 yielded an Rf value of 0.36.

H NMR ($CDCl_3$) δ 0.78 - 1.90 (brm, 38H, CH_2),
2.08 - 2.26 (s, 9H, three CH_3),
2.56 (t, 2H, CH_2)
3.68 (s, 9H, $-N^+(CH_3)_3$),
4.10 (s, 2H, CH_2),
4.19 (s, 1H, CH_2).

Elemental analysis was conducted and the results were compared to the theoretical results which would be expected from a compound with the formula $C_{34}H_{62}NO_2I$.

Theoretical:	C 63.43	H 9.71	N 2.18
Actual results:	C 63.20	H 9.52	N 2.26

While the invention has been described in terms of the preferred embodiments of the invention, those skilled in the art will recognize that the invention may be practiced with modification within the spirit and scope of the appended claims.

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CLAIMS

We claim:

Claim 1. A method for treating ocular melanoma in a patient in need thereof, comprising:

administering to said patient a preparation comprising a sufficient quantity of at least one anionic tocopherol ether or ester.

Claim 2. The method of claim 1 wherein said anionic tocopherol ether or ester is a PEG ester of a anionic tocopherol ether or ester.

Claim 3. The method of claim 1 wherein said anionic tocopherol ether or ester is a salt.

Claim 4. The method of claim 3 wherein said anionic tocopherol ether or ester is a tris salt.

Claim 5. The method of claim 1 wherein said anionic tocopherol is an ether selected from the group consisting of TSE, TSE-T, and TSE-PEG or an ester selected from the group consisting of α-TS, α-TS-tris, α-T-MS, δ-TS, γ-TS-tris, 2,2-Dim-TG, 2,2-Dim-TS, TS-PEG, TRF-S, and TRF-ST.

Claim 6. A method for treating cancer selected from the group consisting of liver cancer, leukemia, breast cancer, prostate cancer, ocular melanoma, cutaneous melanoma, colon cancer, and lung cancer in a patient in need thereof, comprising:

administering to said patient a preparation comprising a sufficient quantity of at least one cationic tocopherol ether or ester.

Claim 7. The method of claim 6 wherein said cationic tocopherol ether or ester is a free base.

Claim 8. The method of claim 6 wherein said cationic tocopherol ether or ester is a salt.

Claim 9. The method of claim 6 wherein said cationic tocopherol ether or ester is an amine.

Claim 10. The method of claim 9 wherein said amine is selected from the group consisting of quaternary and tertiary amines.

Claim 11. The method of claim 6 wherein said cationic tocopherol ester is selected from the group consisting of T-DMAB-Q, T-DMAB-T and said ether is T-DMAE-ether.

Claim 12. A method for treating cancer selected from the group consisting of liver cancer, prostate cancer, ocular melanoma, cutaneous melanoma, colon cancer, and lung cancer in a patient in need thereof, comprising:
administering to said patient a preparation comprising a sufficient quantity of at least one anionic cholesterol ether or ester.

Claim 13. The method of claim 12 wherein said anionic cholesterol ether or ester is a PEG ester of an anionic cholesterol ether or ester.

Claim 14. The method of claim 12 wherein said anionic cholesterol ether or ester is a salt.

Claim 15. The method of claim 14 wherein said anionic cholesterol ether or ester is a tris salt.

Claim 16. The method of claim 12 wherein said anionic cholesterol ether or ester is selected from the group consisting of CS tris, CS-PEG, γ-CSE-tris, and choles-h-p.

Claim 17. A method for treating cancer selected from the group consisting of liver cancer, leukemia, breast cancer, prostate cancer, ocular melanoma, cutaneous melanoma, colon cancer, and lung cancer in a patient in need thereof, comprising:
administering to said patient a preparation comprising a sufficient quantity of at least one cationic cholesterol ether or ester.

Claim 18. The method of claim 17 wherein said cationic cholesterol ether or ester is a free base.

Claim 19. The method of claim 17 wherein said cationic cholesterol ether or ester is a salt.

Claim 20. The method of claim 17 wherein said cationic cholesterol ether or ester is an amine.

Claim 21. The method of claim 20 wherein said amine is selected from the group consisting of quaternary and tertiary amines.

Claim 22. A method for treating cancer selected from the group consisting of liver cancer, leukemia, breast cancer, prostate cancer, ocular melanoma, cutaneous melanoma, colon cancer, and lung cancer in a patient in need thereof, comprising:
administering to said patient a preparation comprising a sufficient quantity of at least one anionic tocotrienol ether or ester.

Claim 23. The method of claim 22 wherein said anionic tocotrienol ether or ester is a PEG ester of an anionic tocotrienol ether or ester.

Claim 24. The method of claim 22 wherein said anionic tocotrienol ether or ester is a salt.

Claim 25. The method of claim 24 wherein said anionic tocotrienol ether or ester is a tris salt.

Claim 26. The method of claim 22 wherein said anionic tocotrienol ether or ester is selected from the group consisting of TRF-S, TRF-S-T, and γ-T3-S.

Claim 27. A method for treating cancer selected from the group consisting of liver cancer, leukemia, breast cancer, prostate cancer, ocular melanoma, cutaneous melanoma, colon cancer, and lung cancer in a patient in need thereof, comprising:
administering to said patient a preparation comprising a sufficient quantity of at least one cationic tocotrienol ether or ester.

Claim 28. The method of claim 27 wherein said cationic tocotrienol ether or ester is a free base.

Claim 29. The method of claim 27 wherein said cationic tocotrienol ether or ester is a salt.

Claim 30. The method of claim 27 wherein said cationic tocotrienol ether or ester is an amine.

Claim 31. The method of claim 30 wherein said amine is selected from the group consisting of quaternary and tertiary amines.

Claim 32. A method for treating cancer selected from the group consisting of liver cancer, leukemia, breast cancer, prostate cancer, ocular melanoma, cutaneous melanoma, colon cancer, and lung cancer in a patient in need thereof, comprising:
administering to said patient a preparation comprising a sufficient quantity of at least one anionic taxol ether or ester.

Claim 33. The method of claim 32 wherein said anionic taxol ether or ester is a PEG ester of an anionic taxol ether or ester.

Claim 34. The method of claim 32 wherein said anionic taxol ether or ester is a salt.

Claim 35. The method of claim 34 wherein said anionic taxol ether or ester is a tris salt.

Claim 36. The method of claim 32 wherein said anionic taxol ether or ester is selected from the group consisting of taxol-S-tris and taxol-DS-tris.

Claim 37. A method for treating cancer selected from the group consisting of liver cancer, breast cancer, prostate cancer, ocular melanoma, cutaneous melanoma, colon cancer, and lung cancer in a patient in need thereof, comprising:
administering to said patient a preparation comprising a sufficient quantity of at least one cationic taxol ether or ester.

Claim 38. The method of claim 37 wherein said cationic taxol ether or ester is a free base.

Claim 39. The method of claim 37 wherein said cationic taxol ether or ester is a salt.

Claim 40. The method of claim 37 wherein said ationic taxol ether or ester is an amine.

Claim 41. The method of claim 40 wherein said amine is selected from the group consisting of quaternary and tertiary amines.

Claim 42. A method for treating cancer selected from the group consisting of: liver cancer, leukemia, breast cancer, prostate cancer, ocular melanoma, cutaneous melanoma, colon cancer, and lung cancer in a patient in need thereof, comprising, administering to said patient a preparation comprising a sufficient quantity of at least one anionic betulinic acid ether or ester.

Claim 43. The method of claim 42 wherein said anionic betulinic acid ether or ester is a PEG ester of an anionic betulinic acid ether or ester.

Claim 44. The method of claim 42 wherein said anionic betulinic acid ether or ester is a salt.

Claim 45. The method of claim 44 wherein said anionic betulinic acid ether or ester is a tris salt.

Claim 46. The method of claim 42 wherein said anionic betulinic acid ether or ester is selected from the group consisting of btl-acid succinate.

Claim 47. A method for treating cancer selected from the group consisting of liver cancer, leukemia, breast cancer, prostate cancer, ocular melanoma, cutaneous melanoma, colon cancer, and lung cancer in a patient in need thereof, comprising: administering to said patient a preparation comprising a sufficient quantity of at least one cationic betulinic acid ether or ester.

Claim 48. The method of claim 47 wherein said cationic betulinic acid ether or ester is a free base.

Claim 49. The method of claim 47 wherein said cationic betulinic acid ether or ester is a salt.

Claim 50. The method of claim 47 wherein said cationic betulinic acid ether or ester is an amine.

Claim 51. The method of claim 50 wherein said amine is selected from the group consisting of quaternary and tertiary amines.

Claim 52. d-α-tocopheryl 2,2-dimethylglutarate.

Claim 53. d-α-tocopheryl 2,2-dimethylglutarate Tris salt.

Claim 54. d-α-tocopheryl β-(N,N-dimethylamino) ethyl ether (free base).

Claim 55. d-α-tocopheryl β-(N,N-dimethylamino) ethyl ether oxalate.

Claim 56. d-α-tocopheryl β-(N,N-dimethylamino) ethyl ether methiodine.

Claim 57. A method for treating cancer selected from the group consisting of liver cancer, prostate cancer, ocular melanoma, cutaneous melanoma, colon cancer, and lung cancer in a patient in need thereof, comprising:

administering to said patient a preparation comprising a sufficient quantity of at least one compound selected from the group consisting of α-TS-tris, α-T-MS, δ-TS, γ-TS-tris, 2,2-Dim TG, 2-2,Dim TS, TS-PEG, TRF-S, TRF-S-T, TSE, TSE-T, and TSE-PEG.

Claim 58. A method for treating breast cancer in a patient in need thereof, comprising:

administering to said patient a preparation comprising a sufficient quantity of at least one leukemia compound selected from the group consisting of α-T-MS, δ-TS, γ-TS-tris, 2,2-Dim TG, 2,2-Dim TS, TS-PEG, TRF-S, TRF-S-T, and TSE-PEG.

Claim 59. A method for treating cancer selected from the group consisting of liver cancer, leukemia, breast cancer, prostate cancer, ocular melanoma, cutaneous melanoma, colon cancer, and lung cancer in a patient in need thereof, comprising:

administering to said patient a preparation comprising a sufficient quantity of a phthalate ester or ether of tocopherol, tocotrienol, taxol, cholesterol or betulinic acid.

Claim 60. The method of claim 59 wherein said ester or ether is a salt.

Claim 61. The method of claim 60 wherein said salt is a tris salt.

Claim 62. The method of claim 59 wherein said pthalate of an ester or ether of tocopherol, tocotrienol, taxol, cholesterol or betulinic acid is a PEG ester of a phthalate ester or ether of tocopherol, tocotrienol, taxol, cholesterol or betulinic acid.

Claim 63. The method of claim 59 wherein said pthalate of an ester or ether of tocopherol, tocotrienol, taxol, cholesterol or betulinic acid is cationic.

Claim 64. The method of claim 63 wherein said cationic phthalate is a free base.

Claim 65. The method of claim 63 wherein said cationic phthalate is a salt.

Claim 66. The method of claim 63 wherein said cationic phthalate is an amine.

Claim 67. The method of claim 66 wherein said amine is selected from the group consisting of quaternary and tertiary amines.

FIGURE 1C

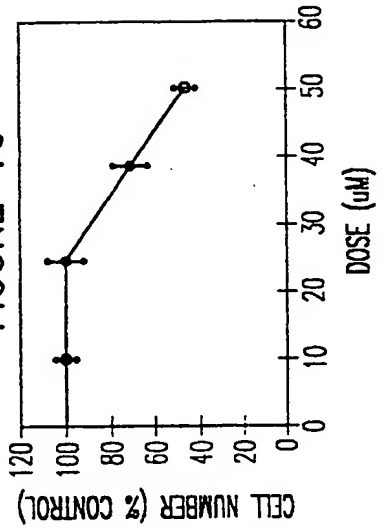


FIGURE 1F

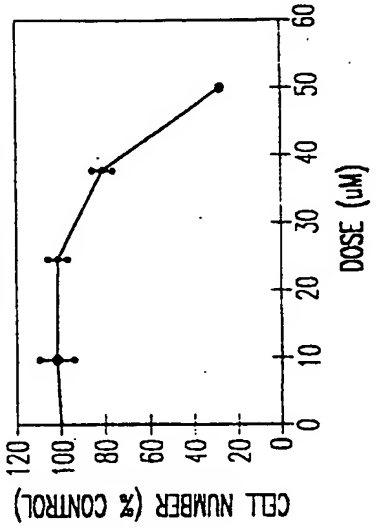


FIGURE 1B

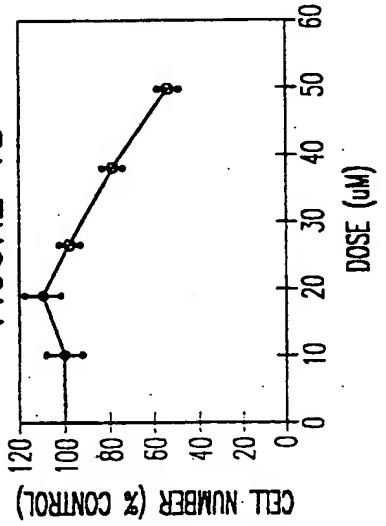


FIGURE 1E

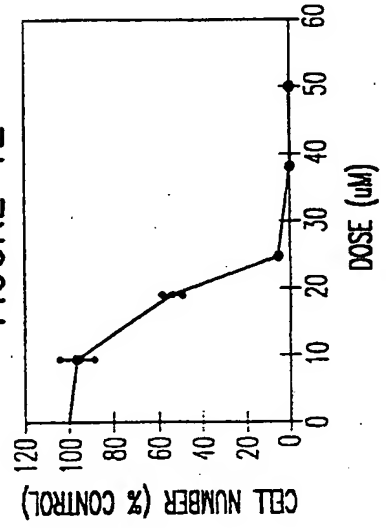


FIGURE 1A

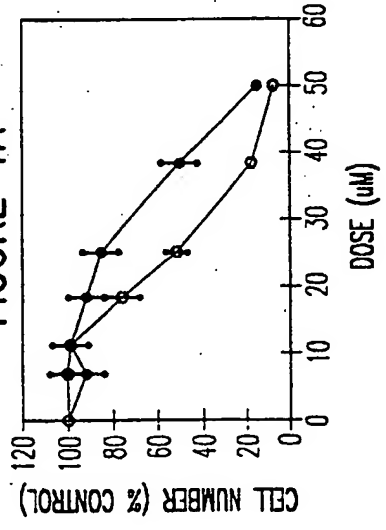


FIGURE 1D

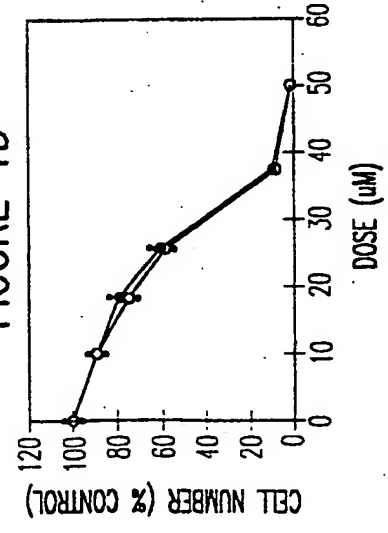


FIGURE 2A

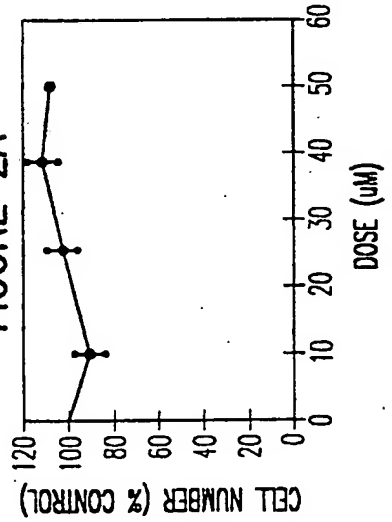


FIGURE 2B

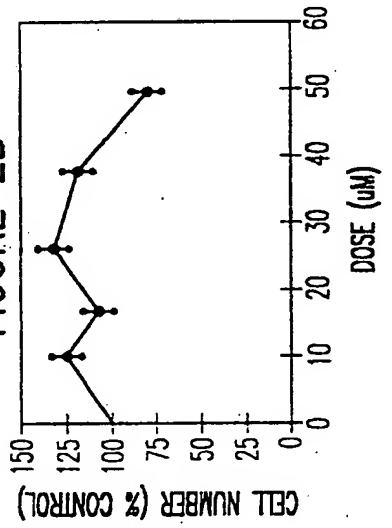


FIGURE 2C

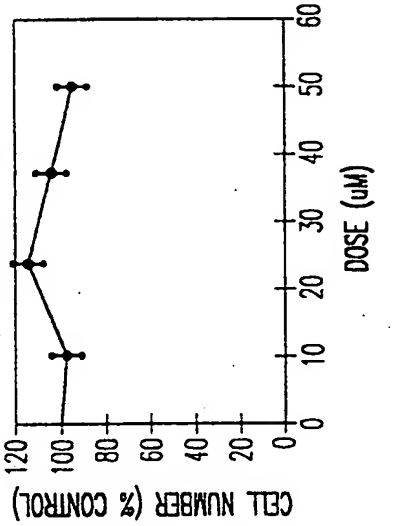


FIGURE 2D

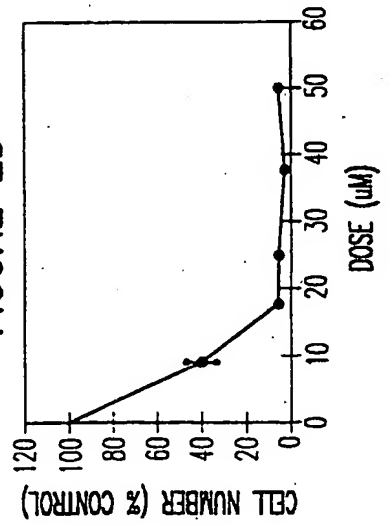


FIGURE 2E

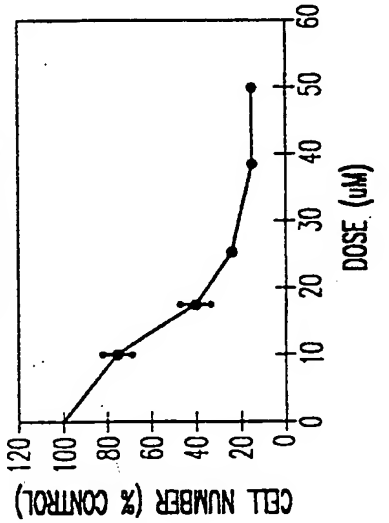


FIGURE 2F

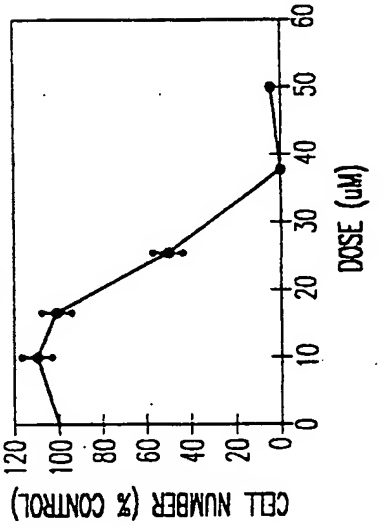


FIGURE 3C

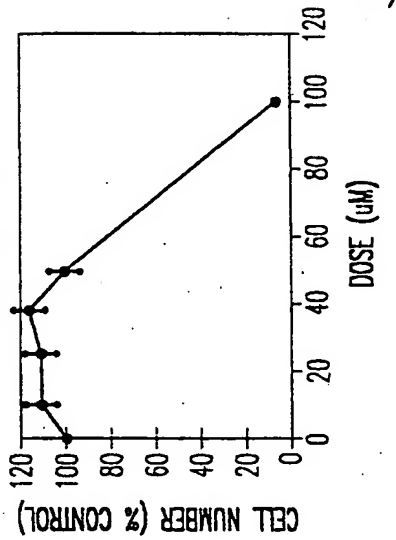


FIGURE 3F

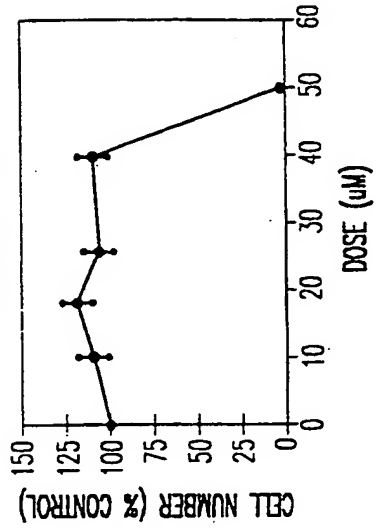


FIGURE 3B

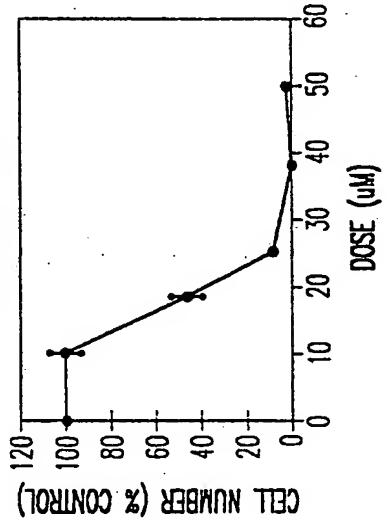


FIGURE 3E

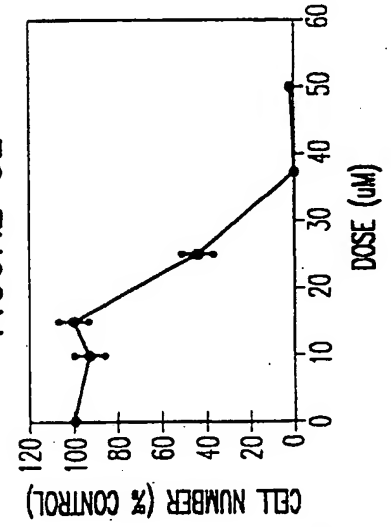


FIGURE 3A

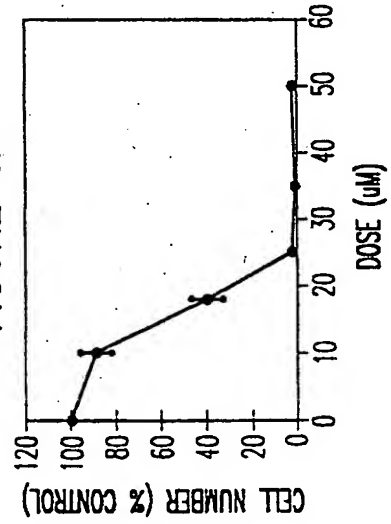


FIGURE 3D

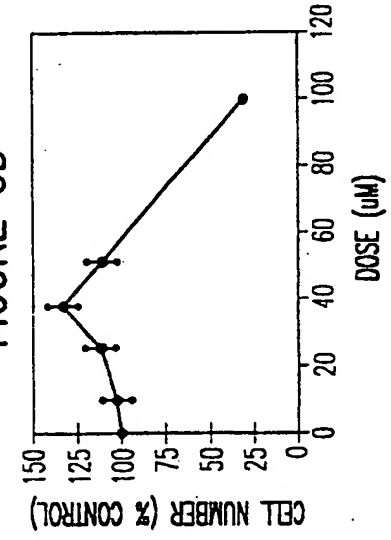


FIGURE 4A

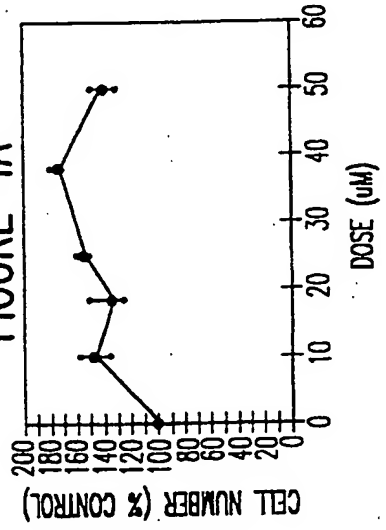


FIGURE 4B

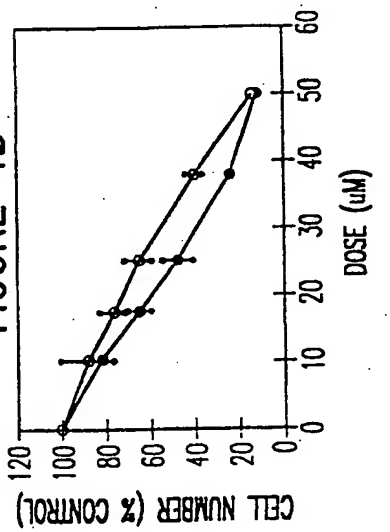


FIGURE 4C

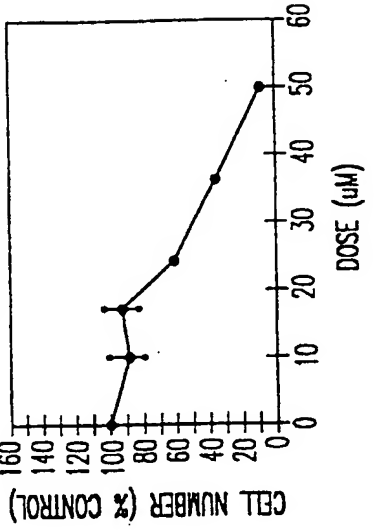


FIGURE 4D

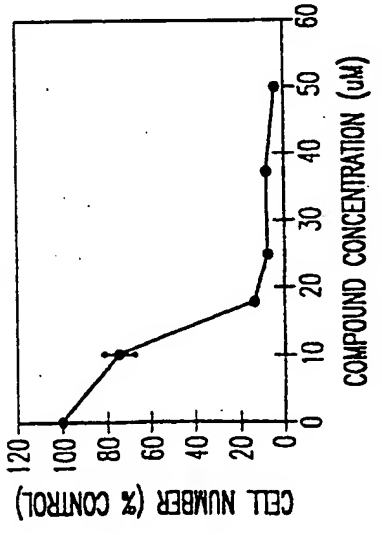


FIGURE 4E

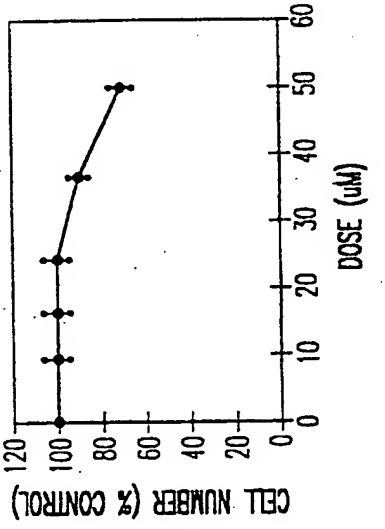


FIGURE 4F

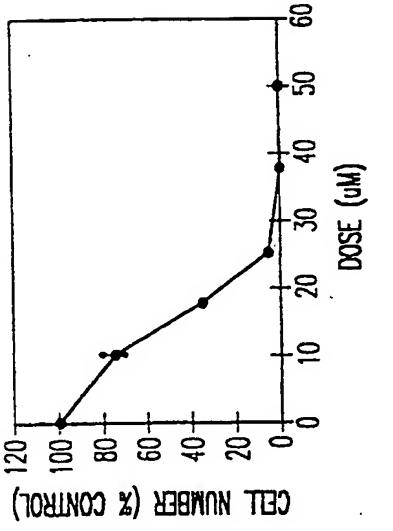


FIGURE 5C

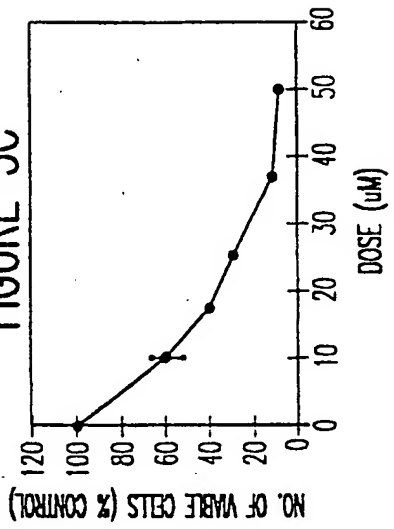


FIGURE 5F

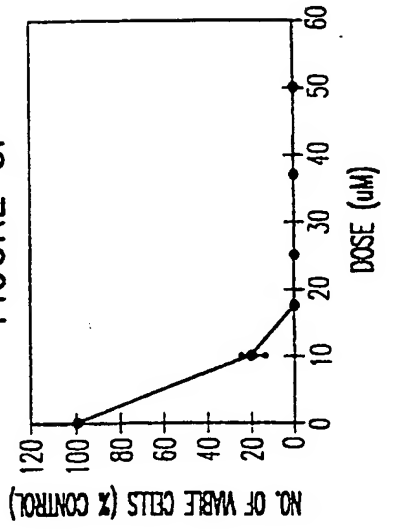


FIGURE 5B

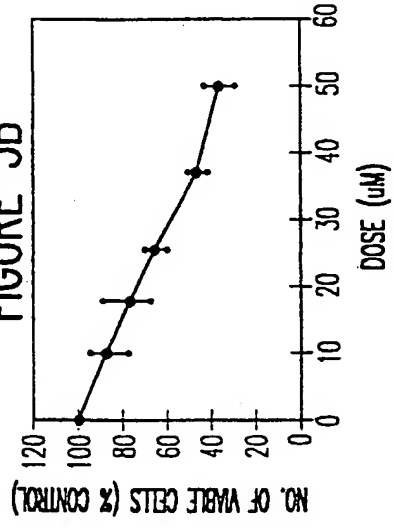


FIGURE 5E

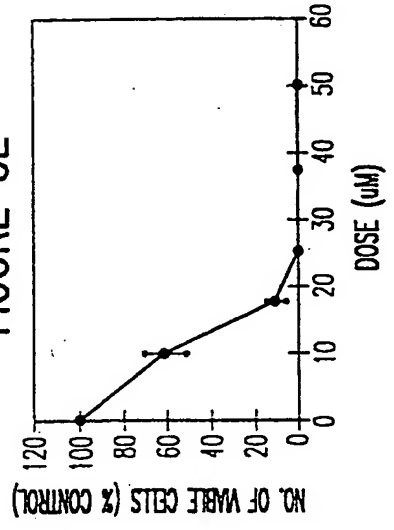


FIGURE 5A

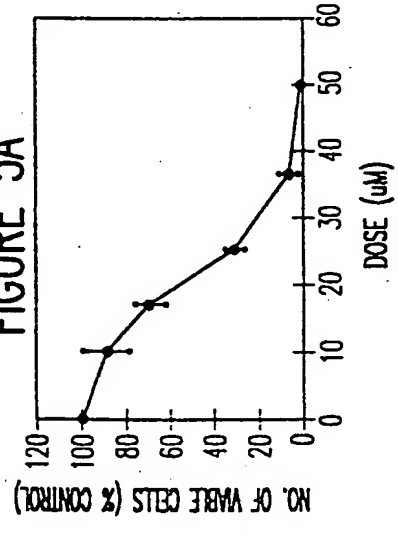


FIGURE 5D

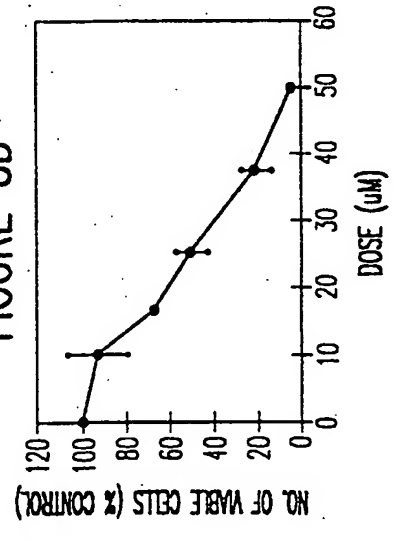
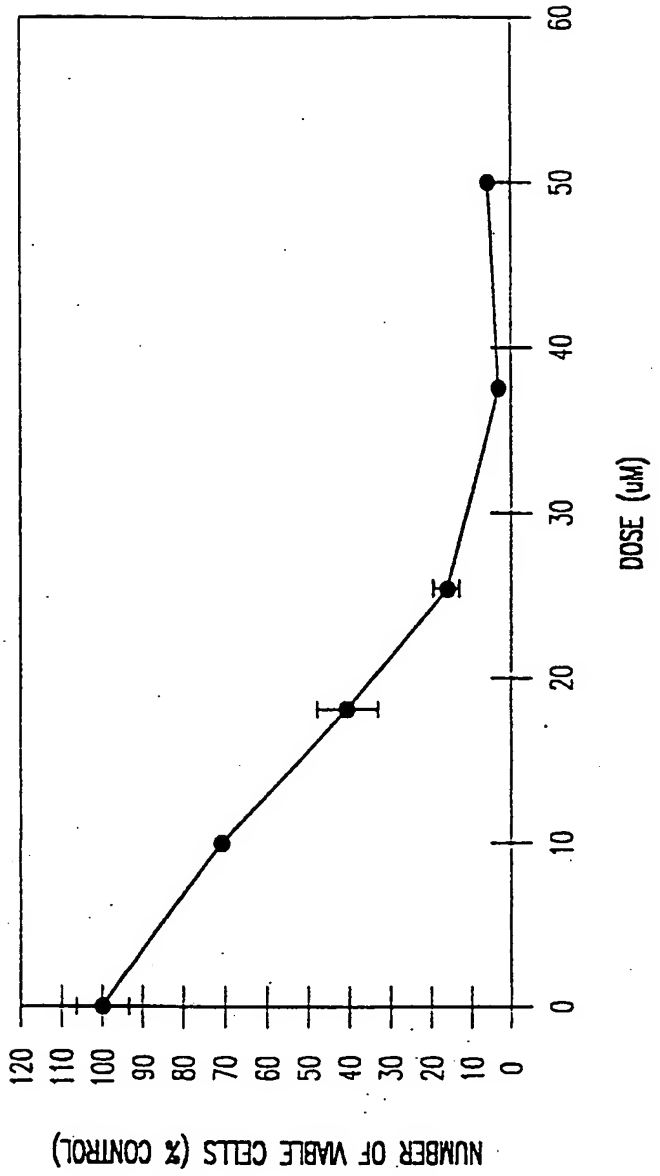


FIGURE 6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100
90
80
70
60
50
40
30
20
10
0
DOSE (uM)
0
10
20
30
40
50
60

FIGURE 7C

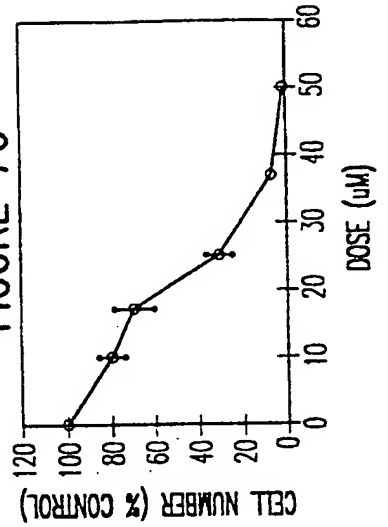


FIGURE 7F

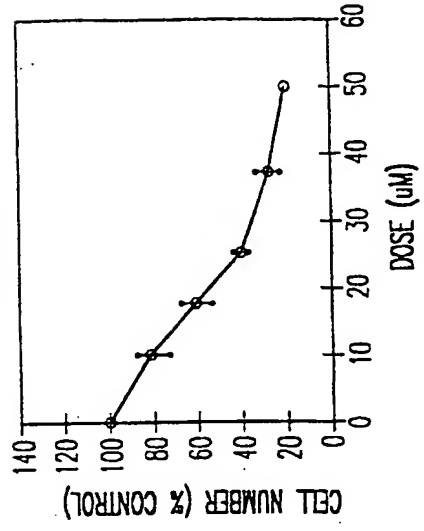


FIGURE 7B

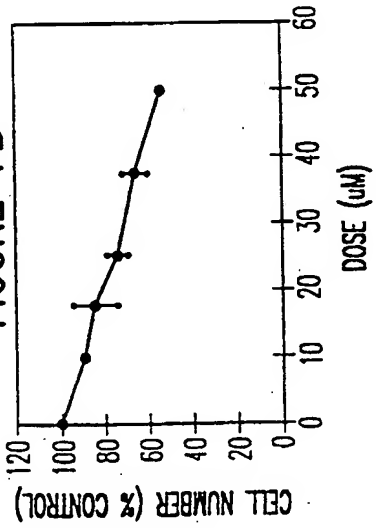


FIGURE 7E

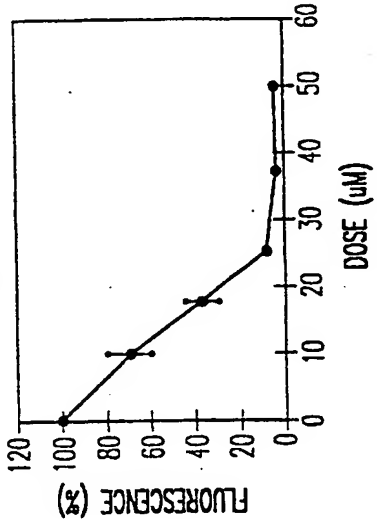


FIGURE 7A

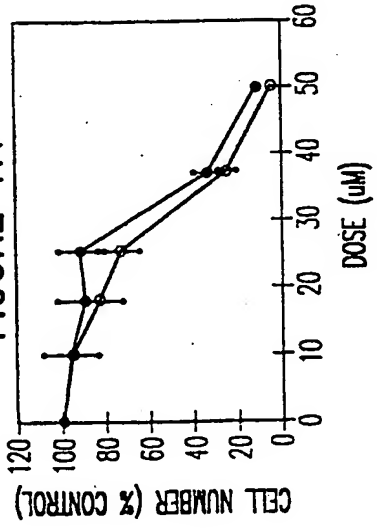


FIGURE 7D

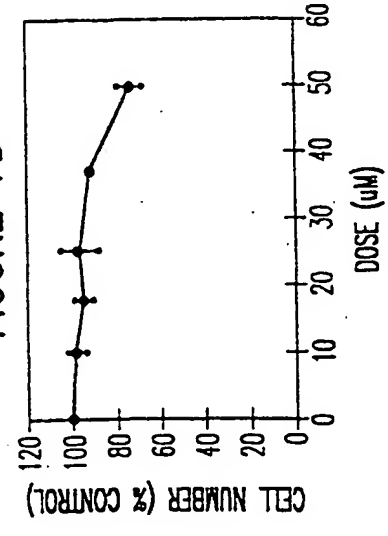


FIGURE 8C

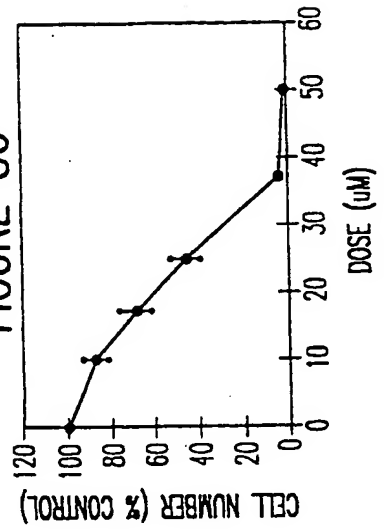


FIGURE 8F

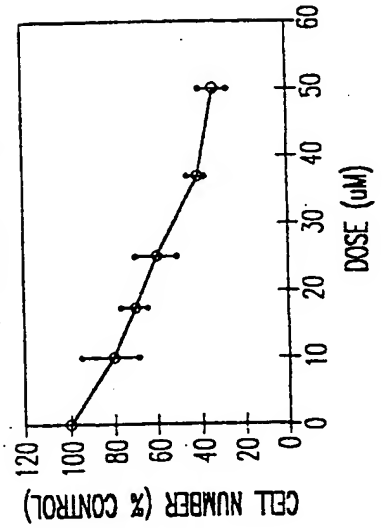


FIGURE 8B

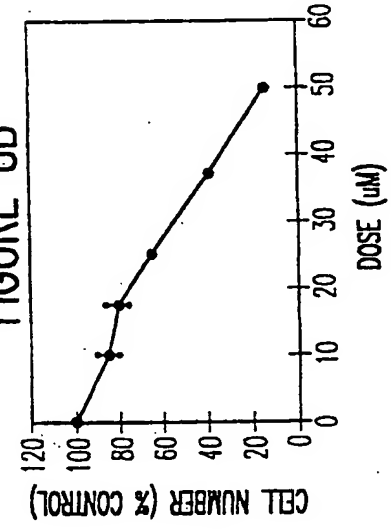


FIGURE 8E

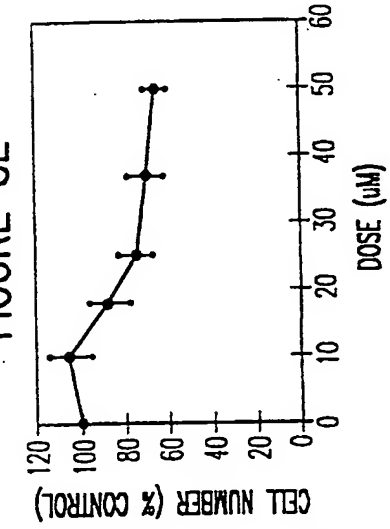


FIGURE 8A

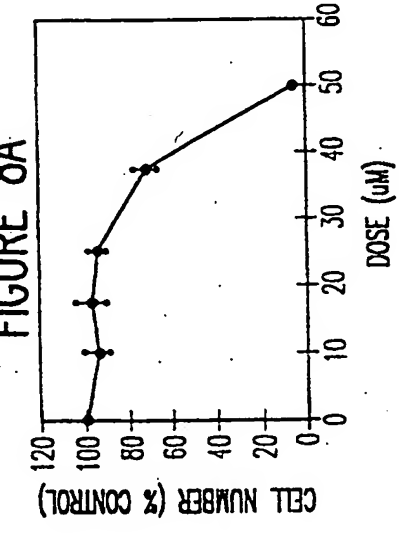


FIGURE 8D

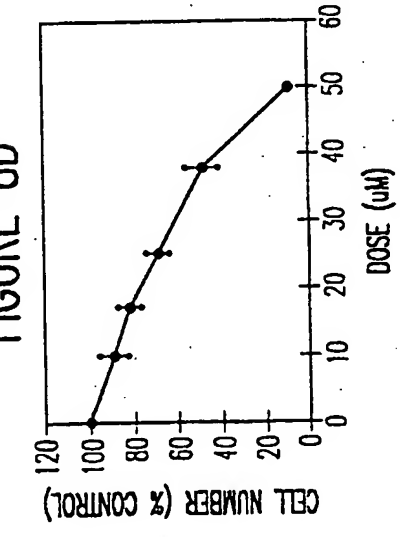


FIGURE 9B

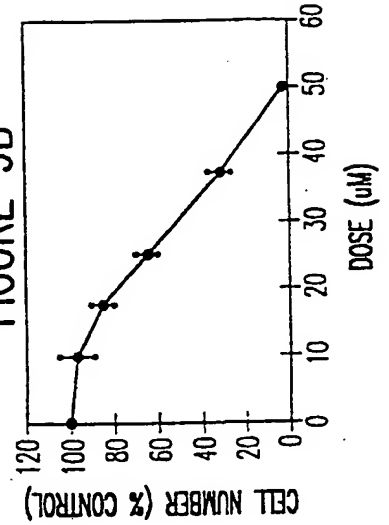


FIGURE 9D

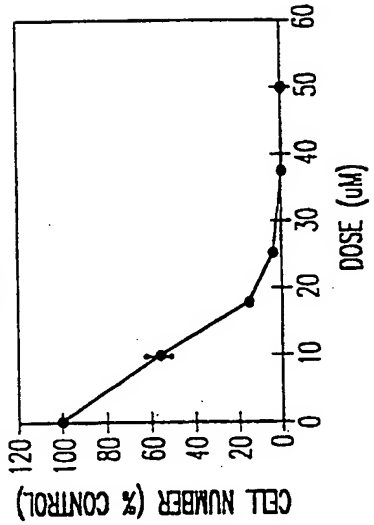


FIGURE 9A

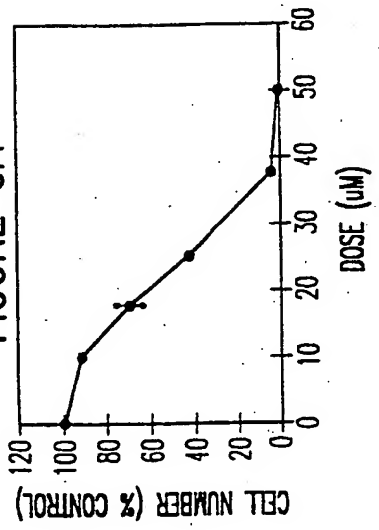
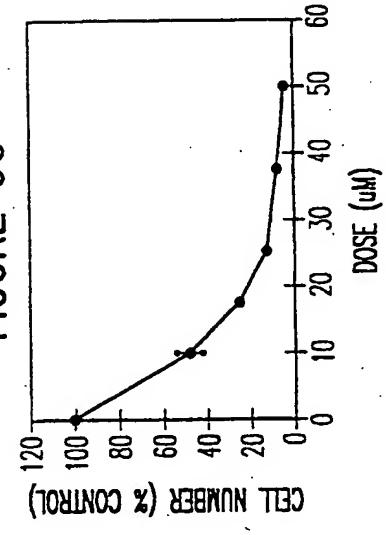


FIGURE 9C



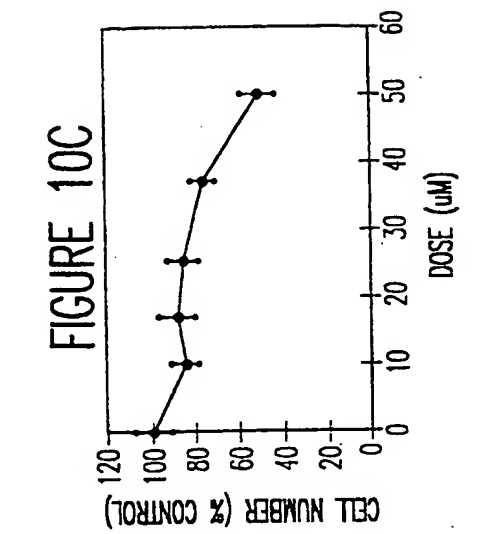


FIGURE 10C
CELL NUMBER (% CONTROL)
DOSE (uM)

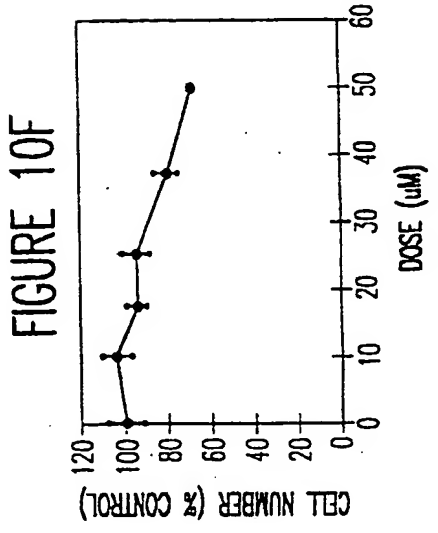


FIGURE 10F
CELL NUMBER (% CONTROL)
DOSE (uM)

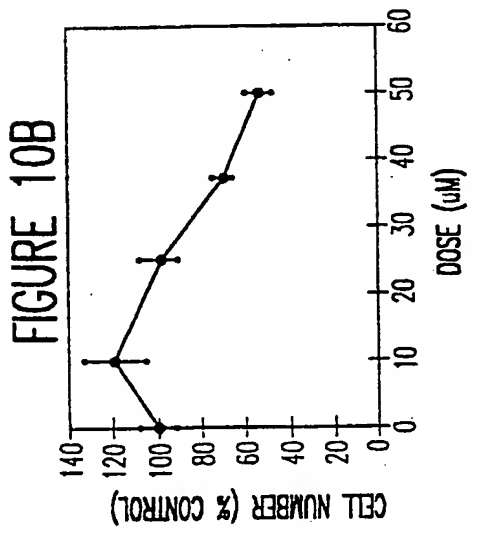


FIGURE 10B
CELL NUMBER (% CONTROL)
DOSE (uM)

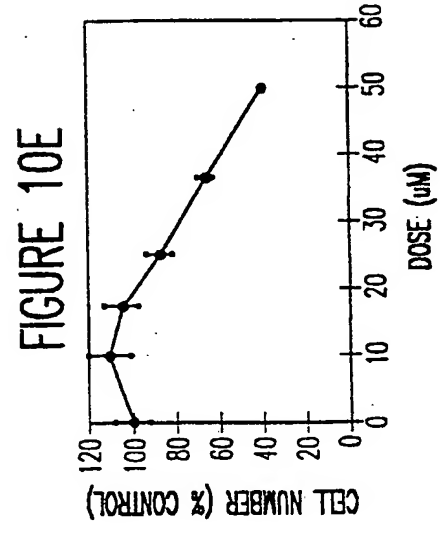


FIGURE 10E
CELL NUMBER (% CONTROL)
DOSE (uM)

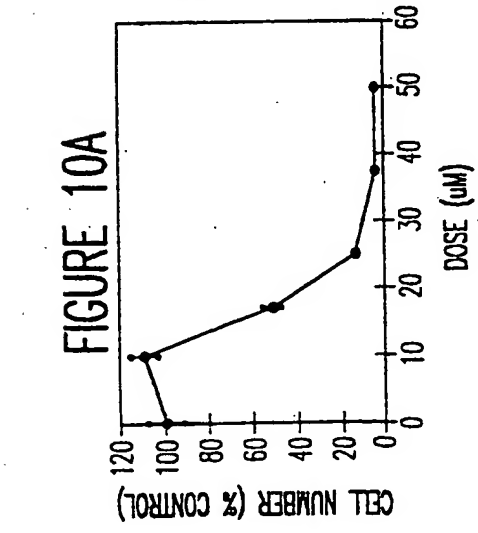


FIGURE 10A
CELL NUMBER (% CONTROL)
DOSE (uM)

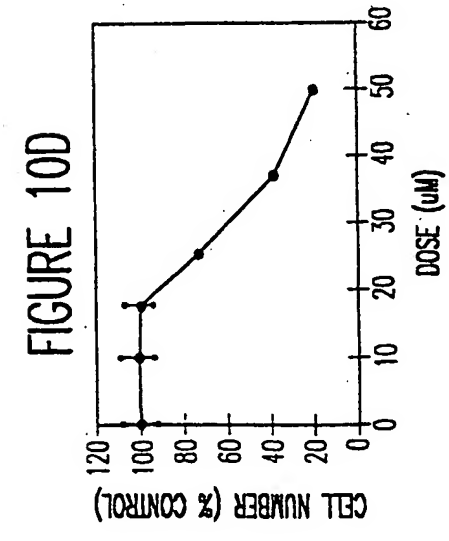


FIGURE 10D
CELL NUMBER (% CONTROL)
DOSE (uM)

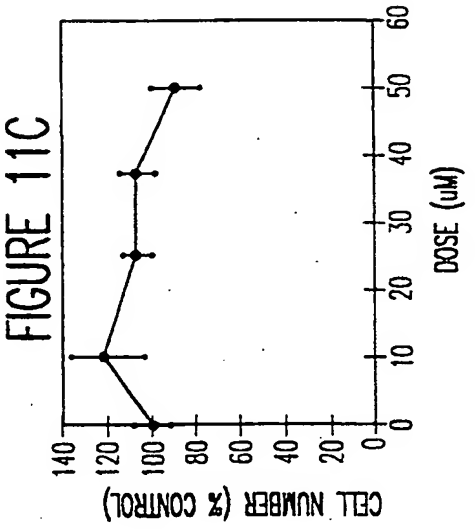


FIGURE 11C
CELL NUMBER (% CONTROL)
DOSE (uM)
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80
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50
60

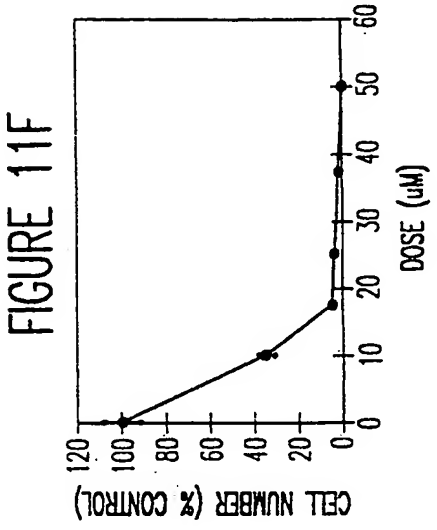
FIGURE 11F
CELL NUMBER (% CONTROL)
DOSE (uM)
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80
100
120
0
10
20
30
40
50
60

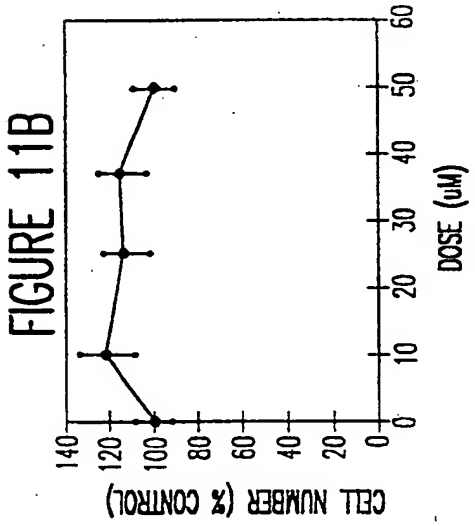
FIGURE 11B
CELL NUMBER (% CONTROL)
DOSE (uM)
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80
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140
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30
40
50
60

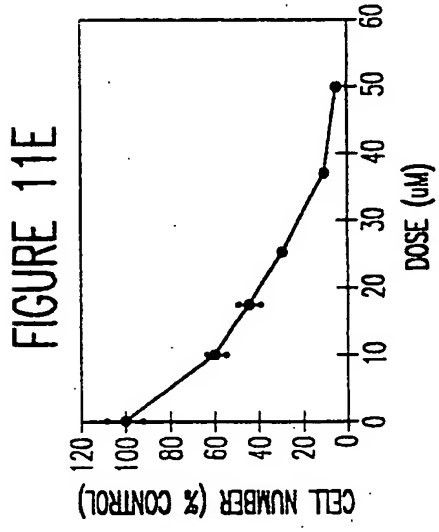
FIGURE 11E
CELL NUMBER (% CONTROL)
DOSE (uM)
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60
80
100
120
0
10
20
30
40
50
60

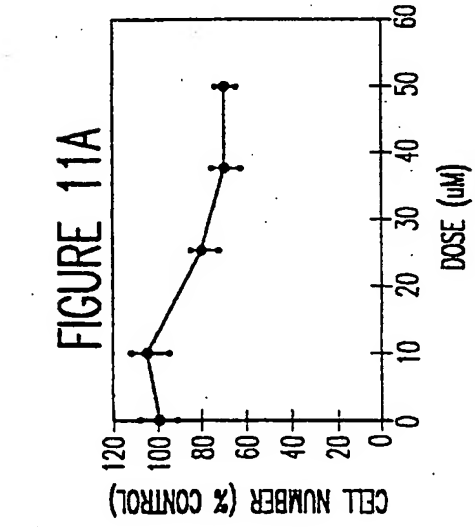
FIGURE 11A
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

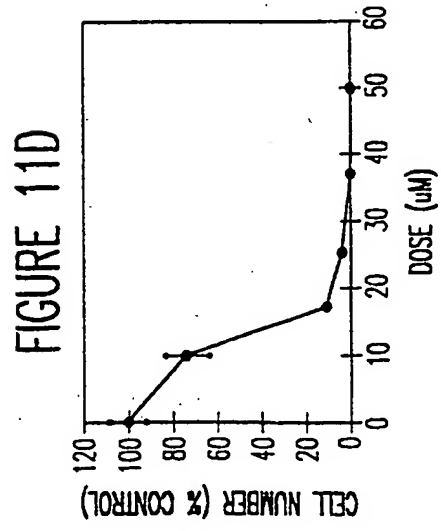
FIGURE 11D
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

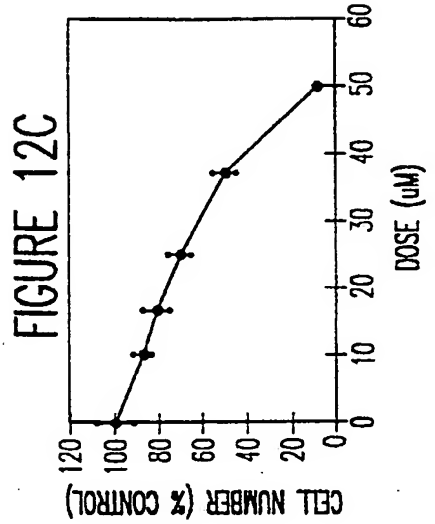
FIGURE 12C
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

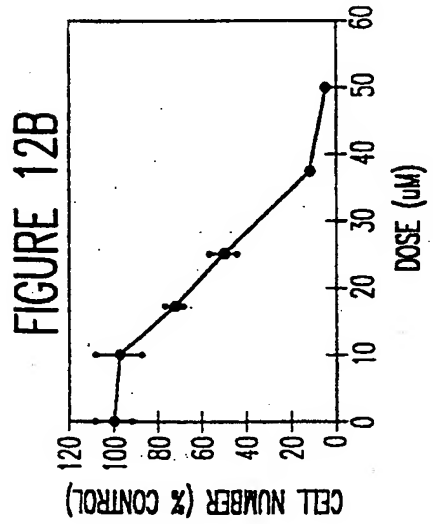
FIGURE 12B
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

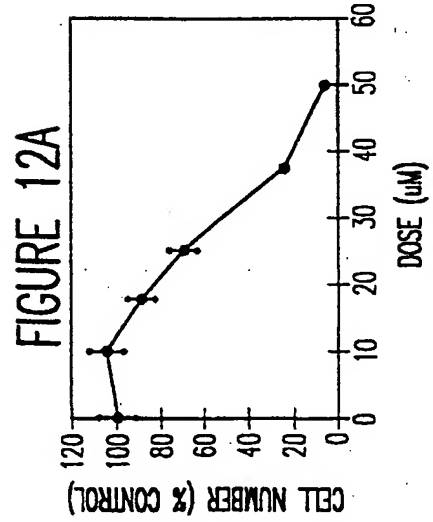
FIGURE 12A
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

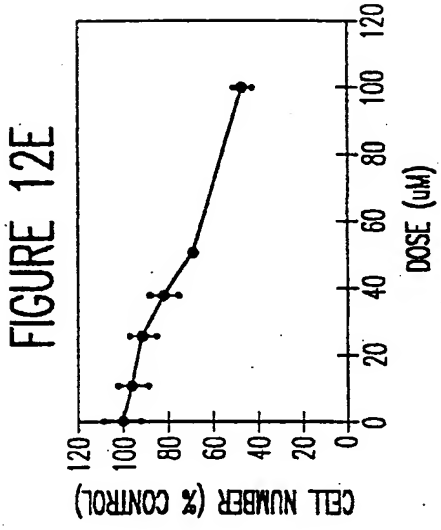
FIGURE 12E
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
20
40
60
80
100
120

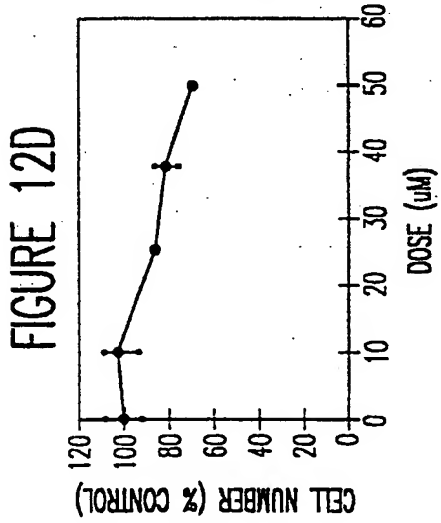
FIGURE 12D
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

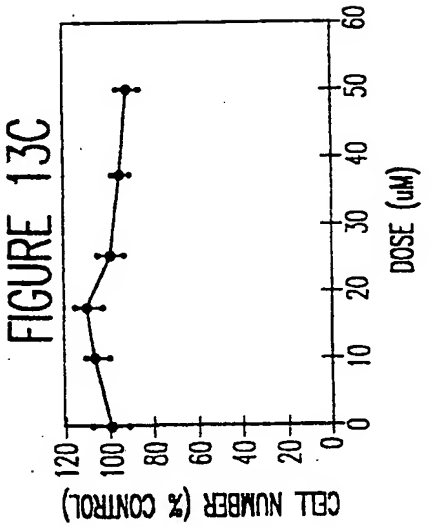
FIGURE 13C
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

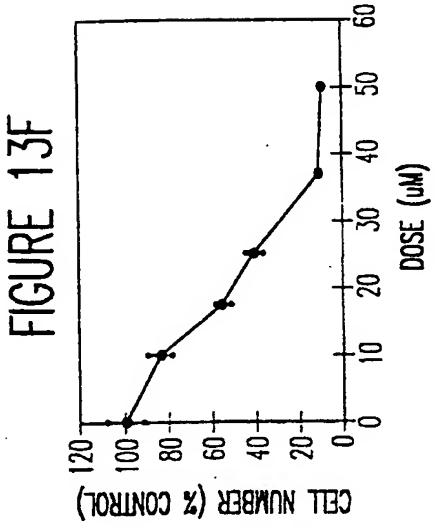
FIGURE 13F
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

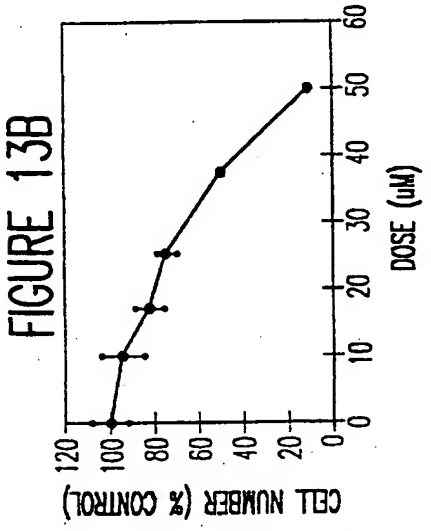
FIGURE 13B
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

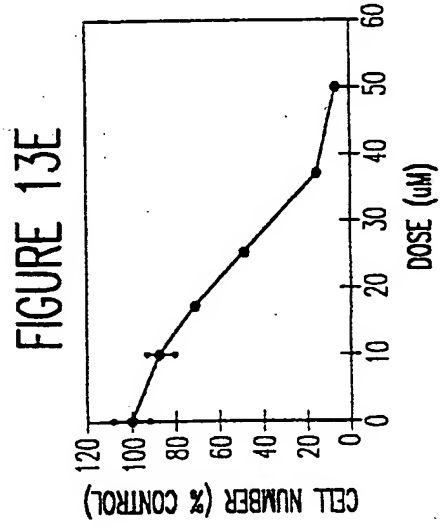
FIGURE 13E
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

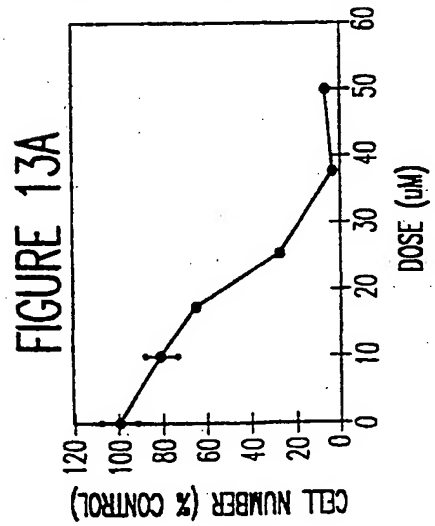
FIGURE 13A
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

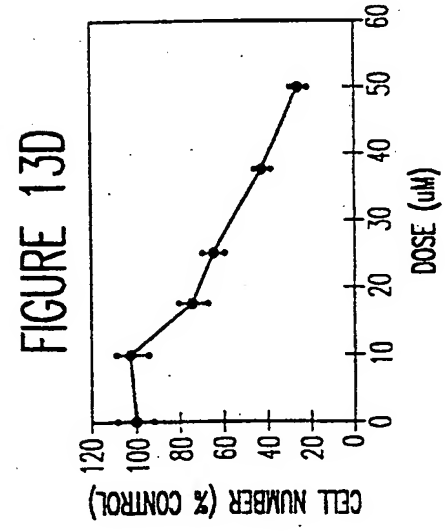
FIGURE 13D
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

FIGURE 14C

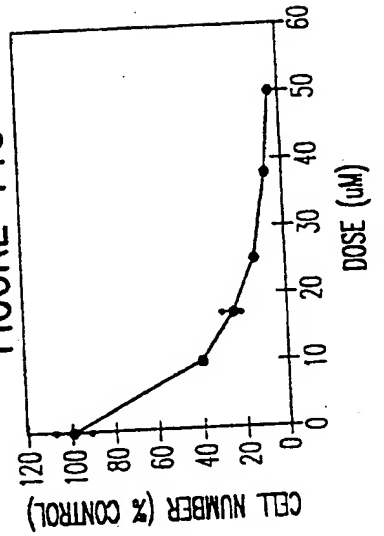

FIGURE 14B

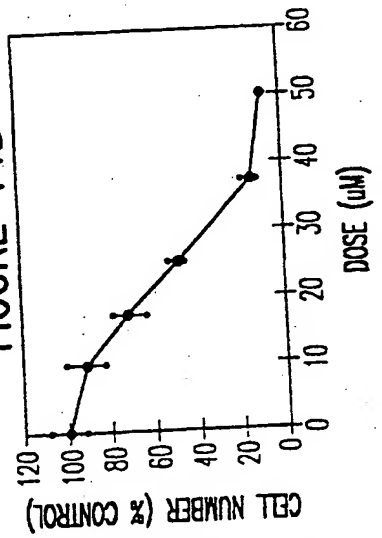

FIGURE 14A

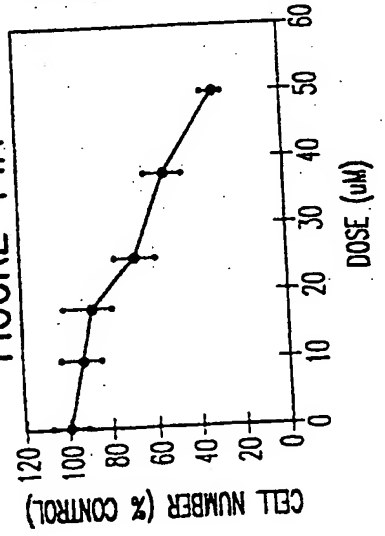

FIGURE 14E

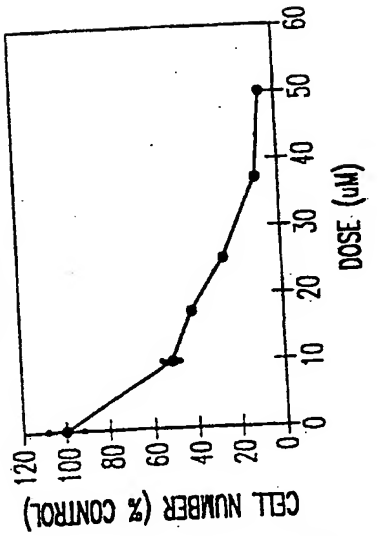

FIGURE 14D

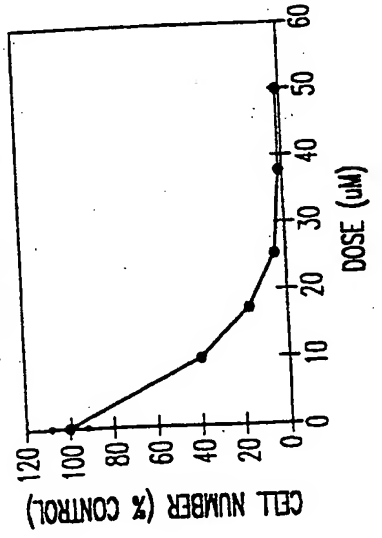

FIGURE 15C

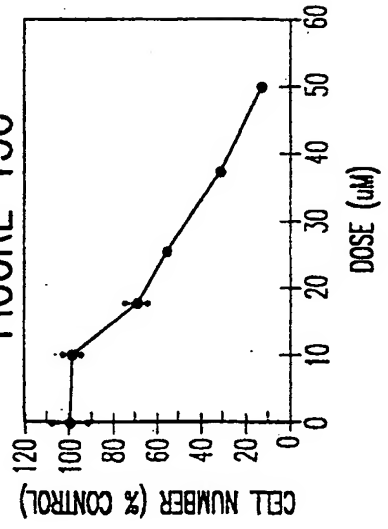

FIGURE 15F

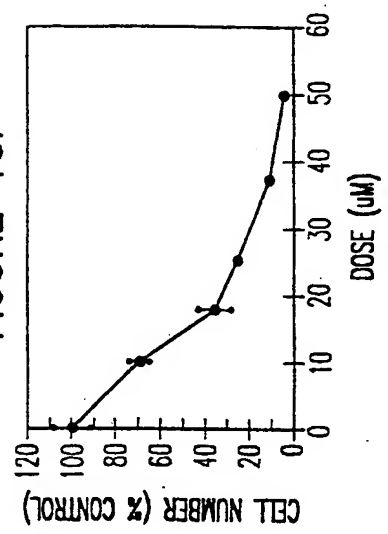

FIGURE 15B

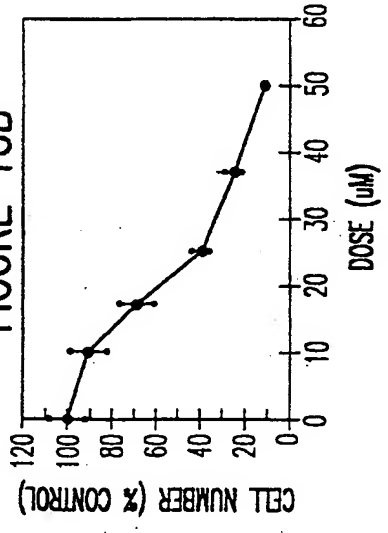

FIGURE 15E

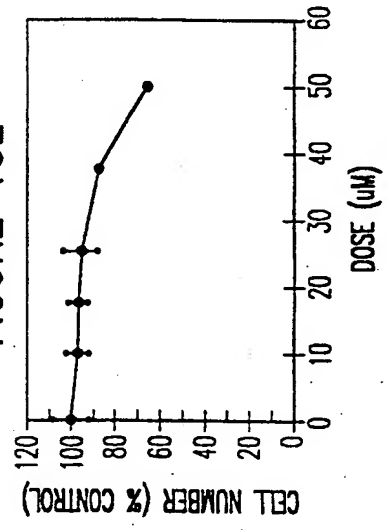

FIGURE 15A

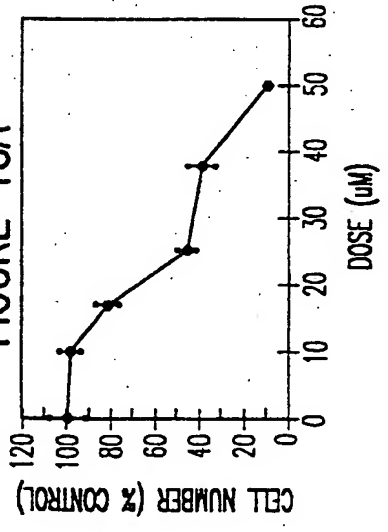

FIGURE 15D

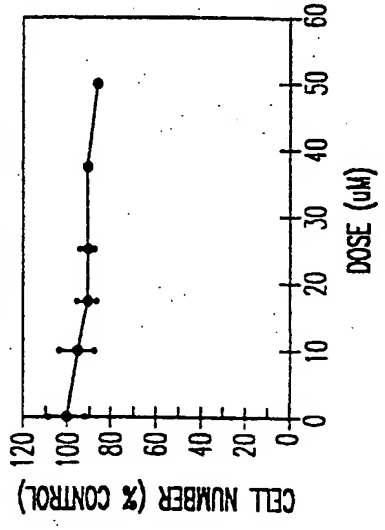

FIGURE 16A

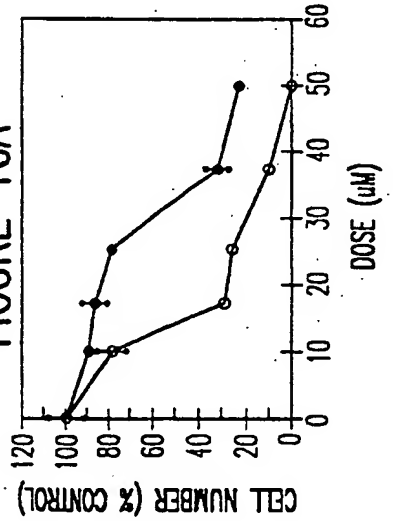

FIGURE 16B

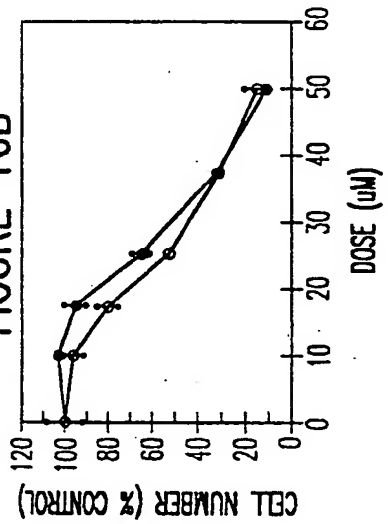

FIGURE 16C

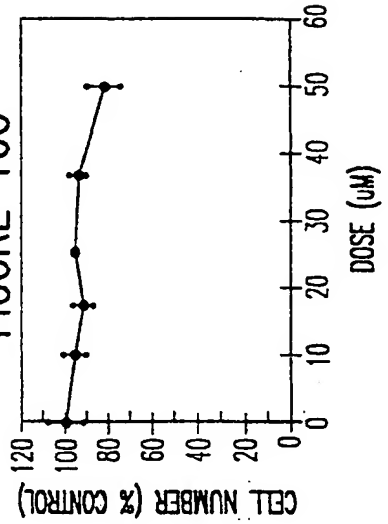

FIGURE 16D

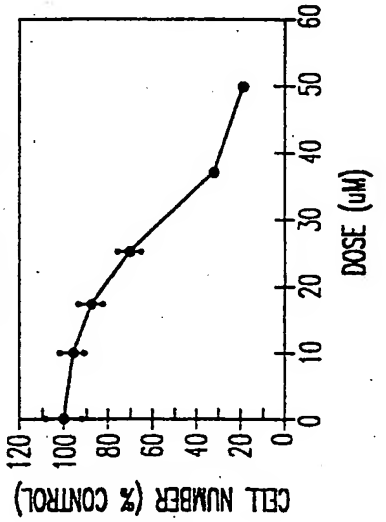

FIGURE 16E

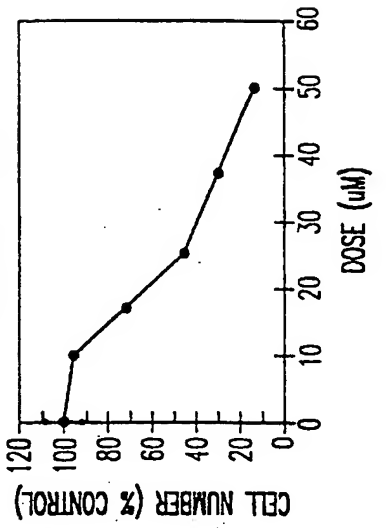

FIGURE 17A

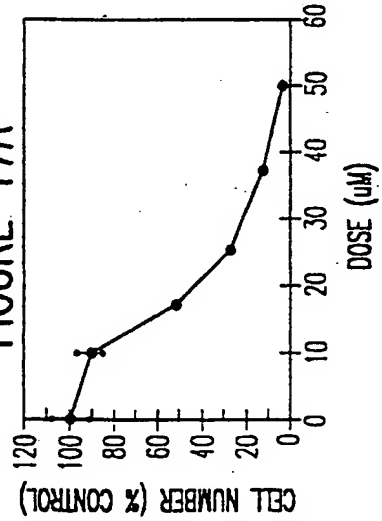

FIGURE 17B

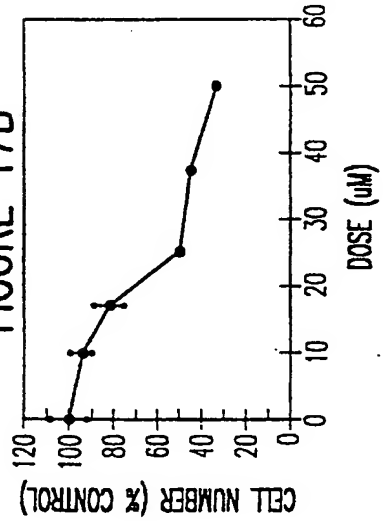

FIGURE 17C

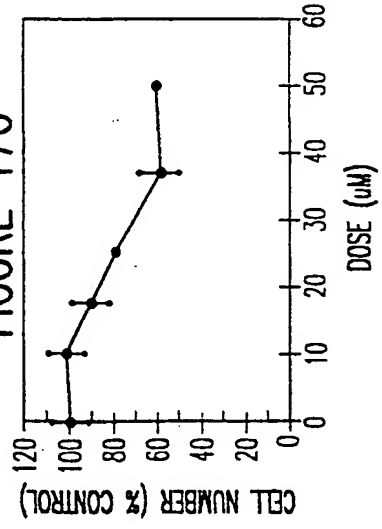

FIGURE 17D

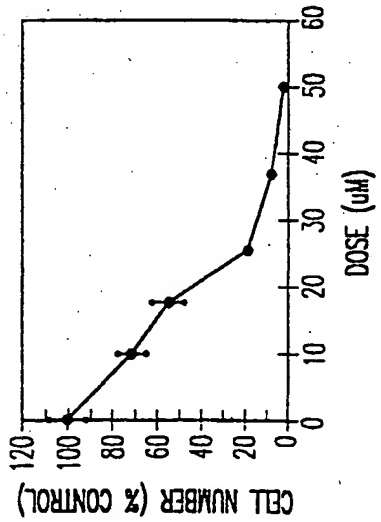

FIGURE 17E

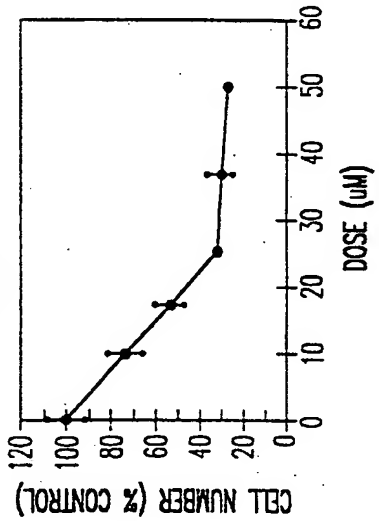

FIGURE 17F

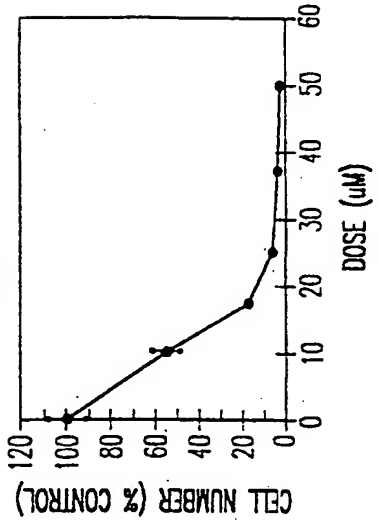

FIGURE 18A

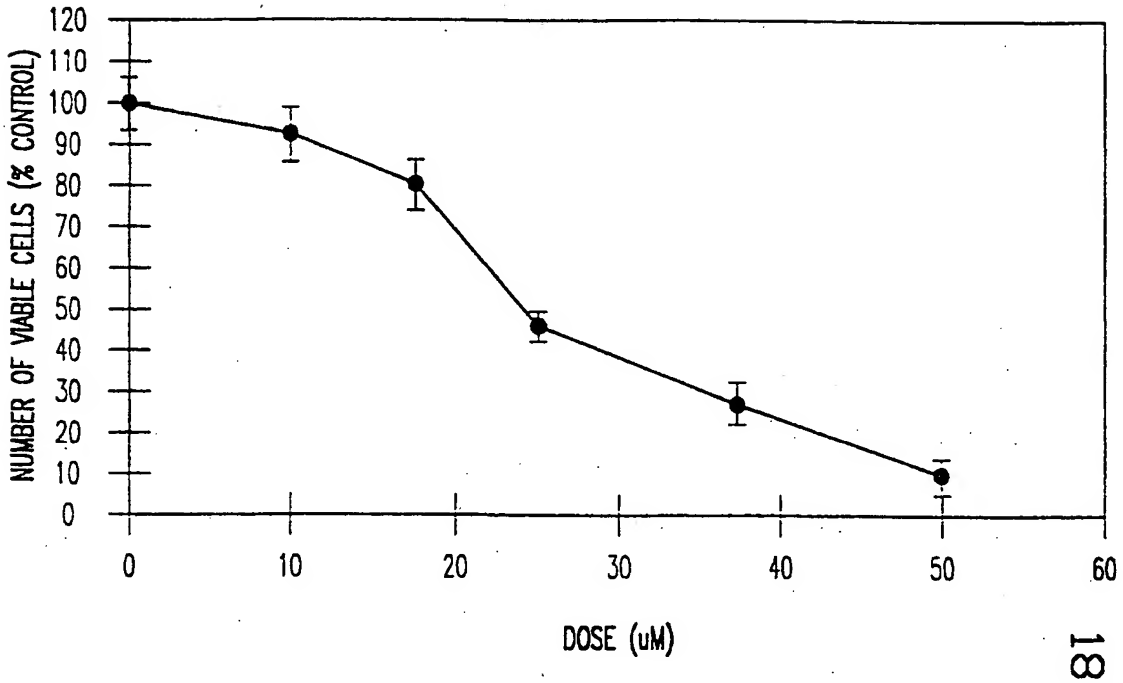

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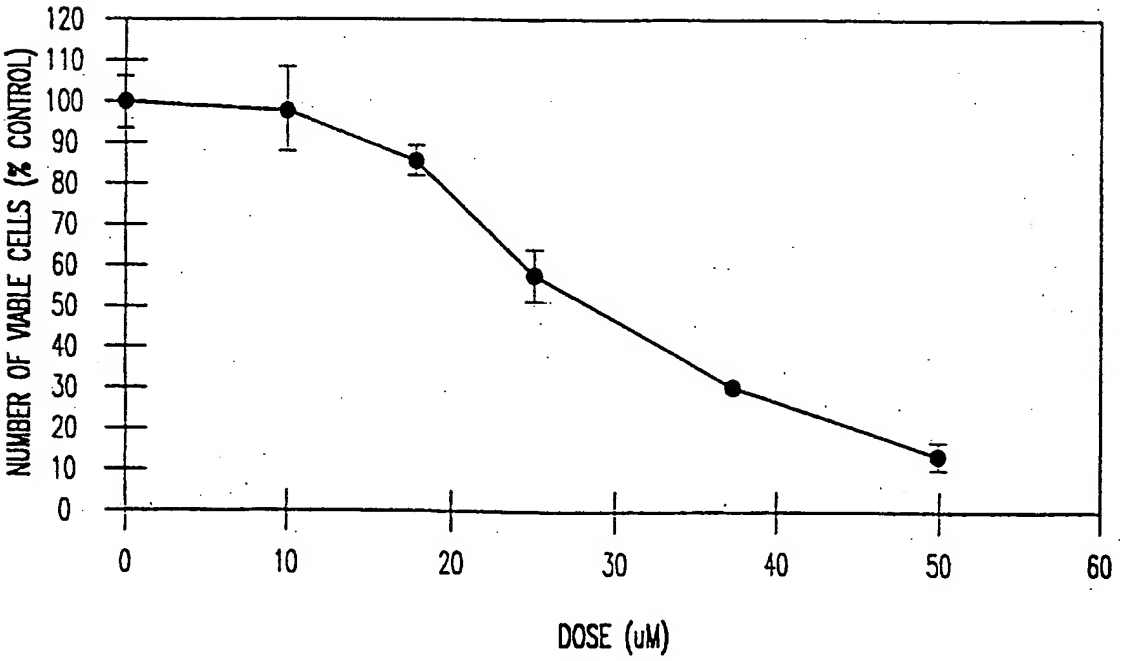
FIGURE 18B



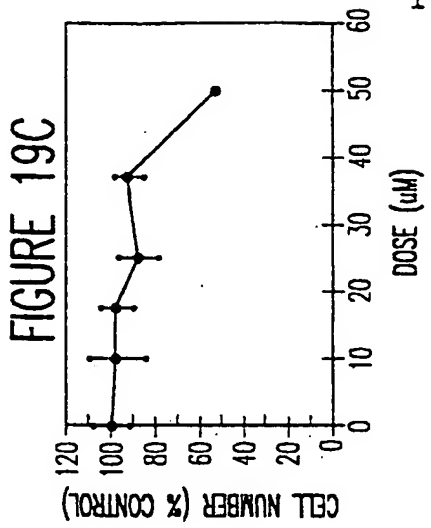


FIGURE 19C
CELL NUMBER (% CONTROL)
DOSE (uM)

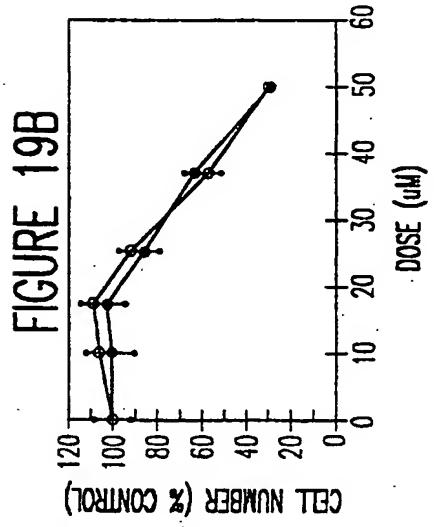


FIGURE 19B
CELL NUMBER (% CONTROL)
DOSE (uM)

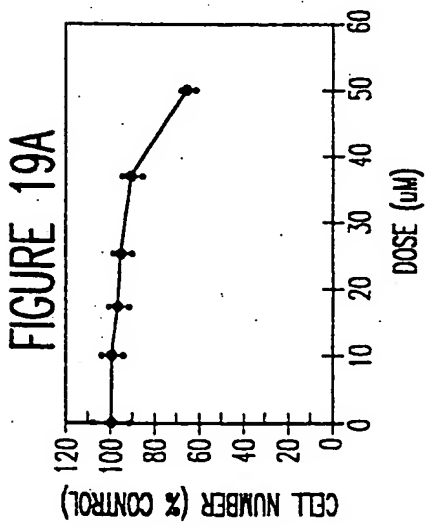


FIGURE 19A
CELL NUMBER (% CONTROL)
DOSE (uM)

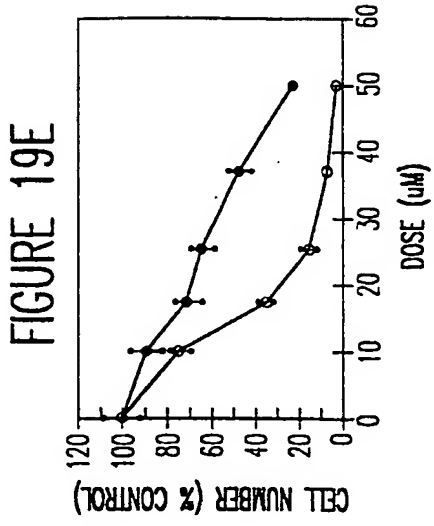


FIGURE 19E
CELL NUMBER (% CONTROL)
DOSE (uM)

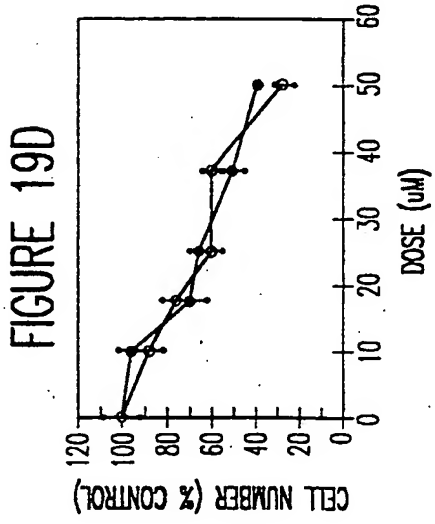


FIGURE 19D
CELL NUMBER (% CONTROL)
DOSE (uM)

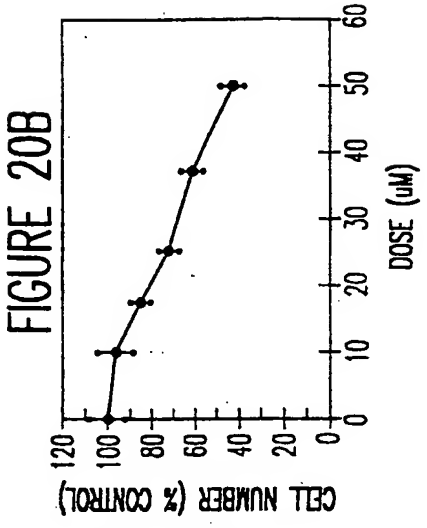


FIGURE 20B
CELL NUMBER (% CONTROL)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
DOSE (uM)

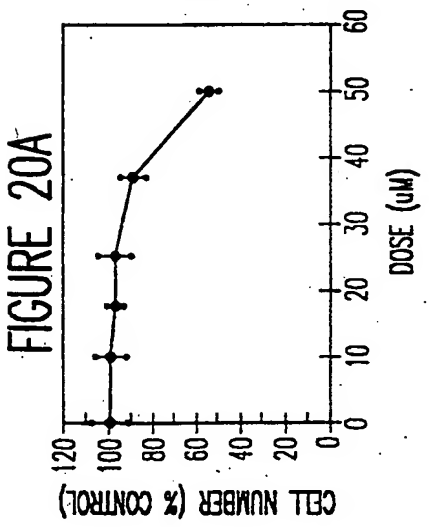


FIGURE 20A
CELL NUMBER (% CONTROL)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
DOSE (uM)

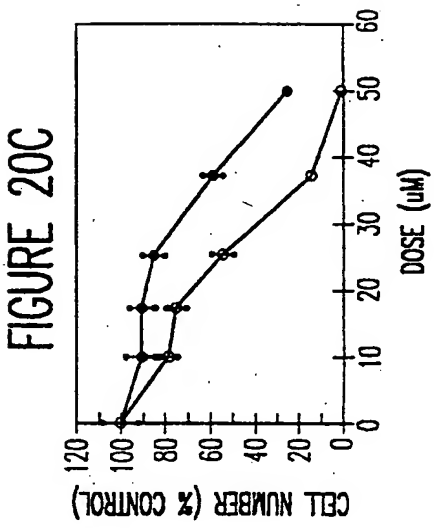


FIGURE 20C
CELL NUMBER (% CONTROL)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
DOSE (uM)

FIGURE 21B

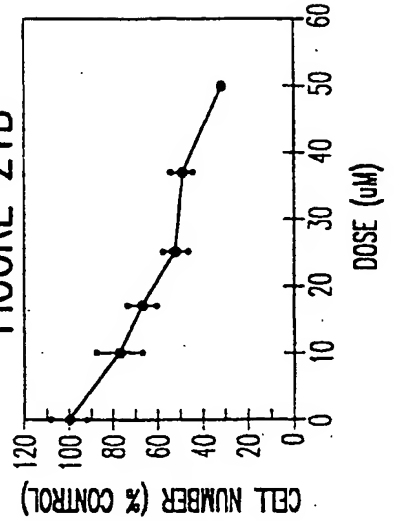


FIGURE 21D

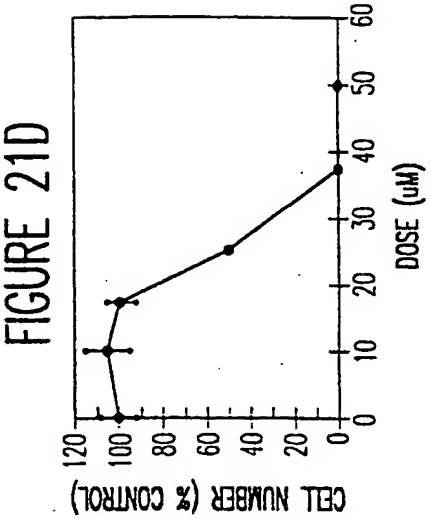


FIGURE 21A

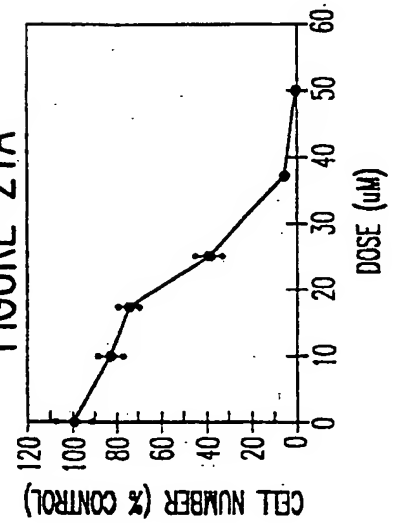


FIGURE 21C

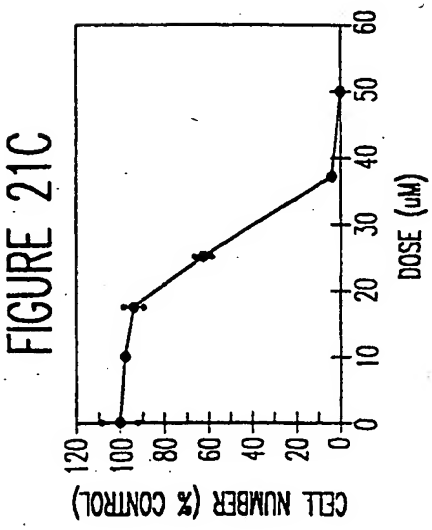


FIGURE 22B

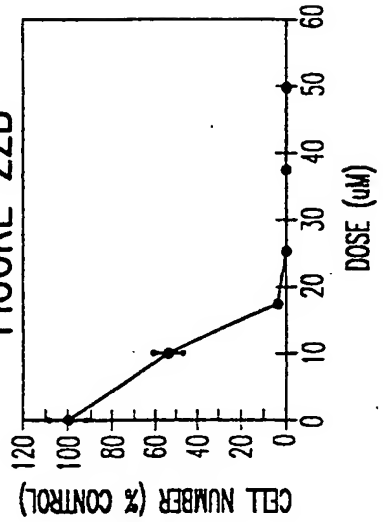


FIGURE 22D

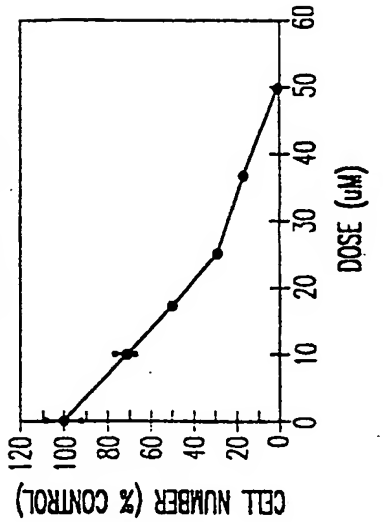


FIGURE 22A

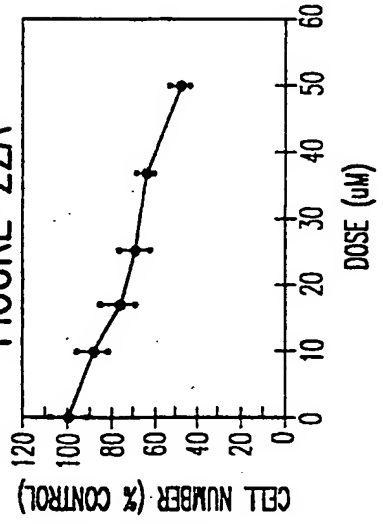


FIGURE 22C

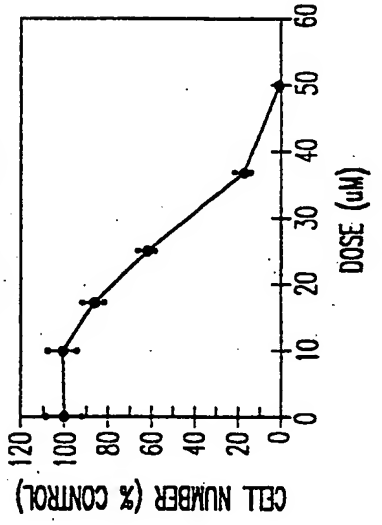


FIGURE 23B

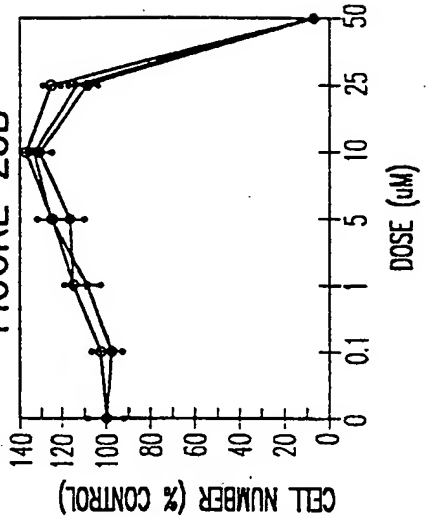


FIGURE 23D

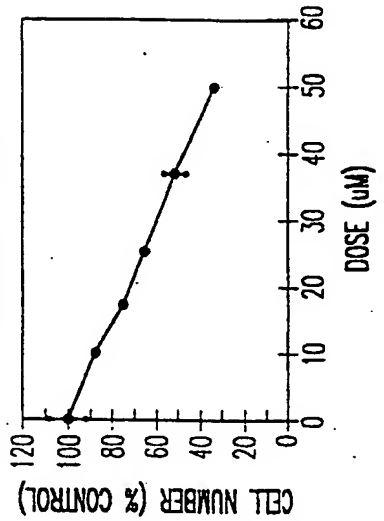


FIGURE 23A

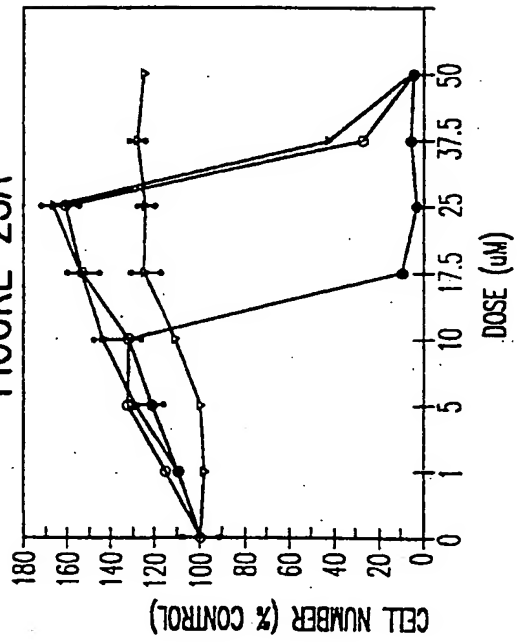


FIGURE 23C

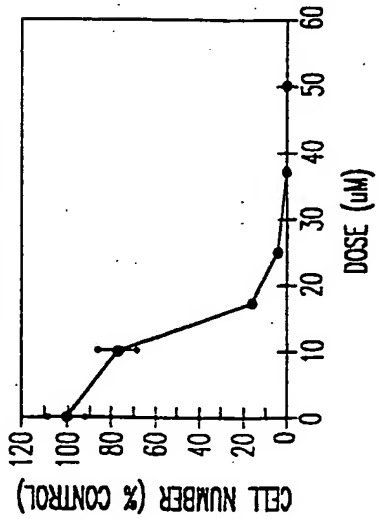


FIGURE 24B

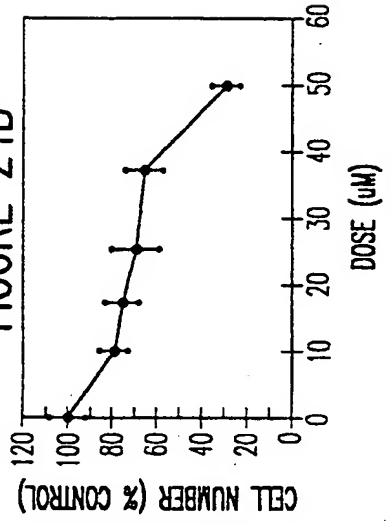


FIGURE 24D

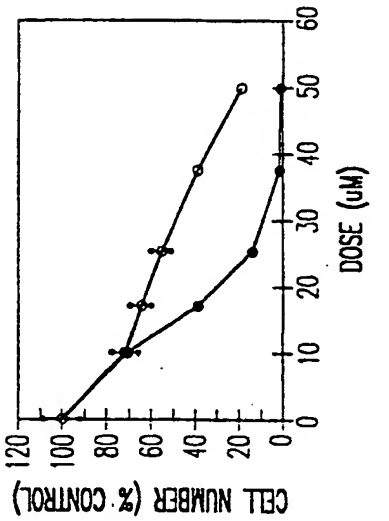


FIGURE 24A

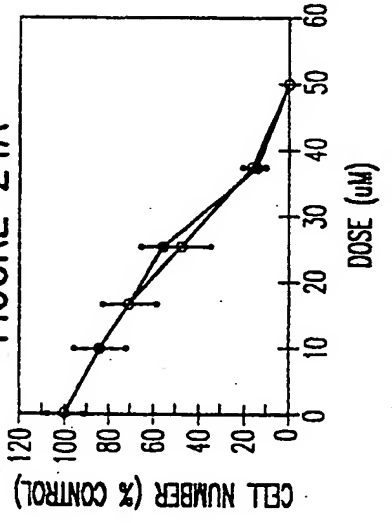


FIGURE 24C

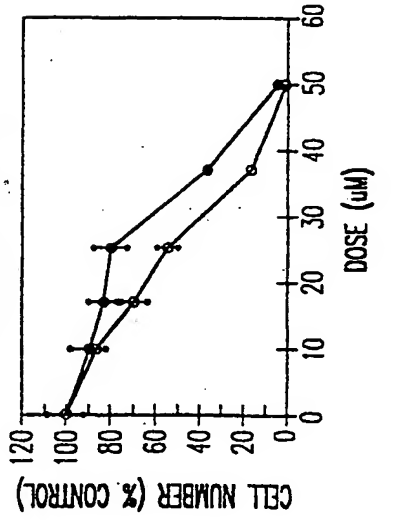


FIGURE 25B

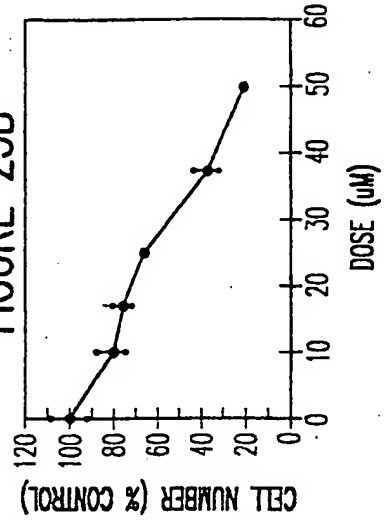


FIGURE 25C

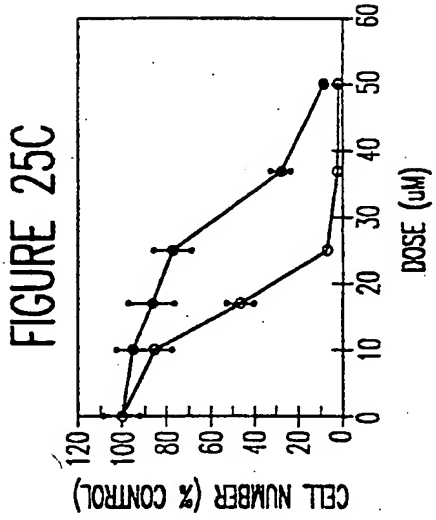


FIGURE 25A

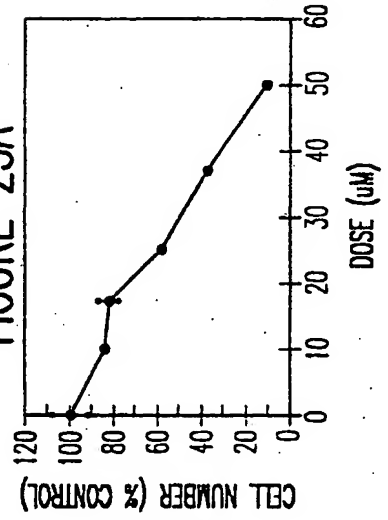


FIGURE 26B

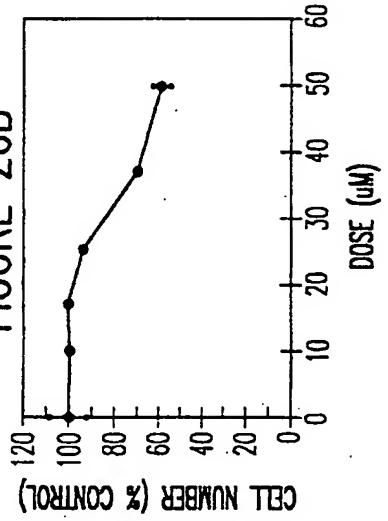


FIGURE 26D

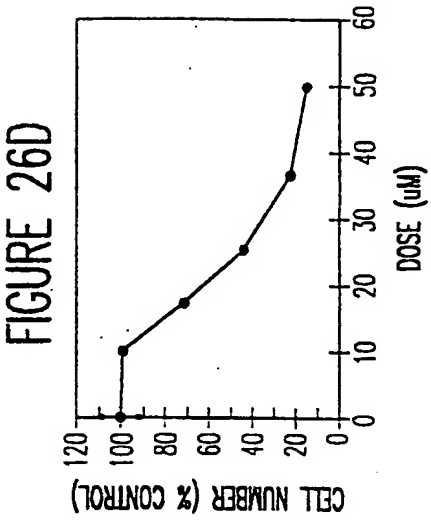


FIGURE 26A

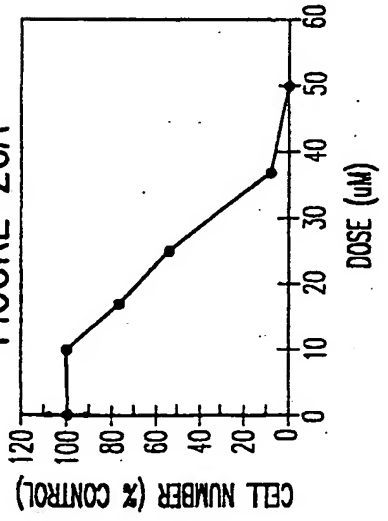
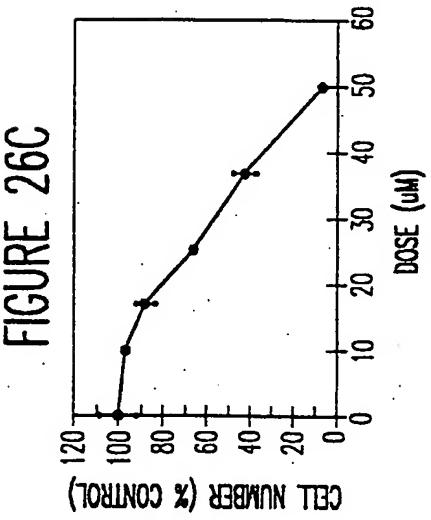


FIGURE 26C



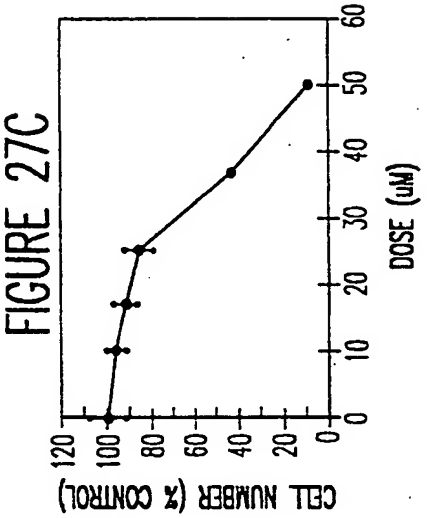


FIGURE 27C
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

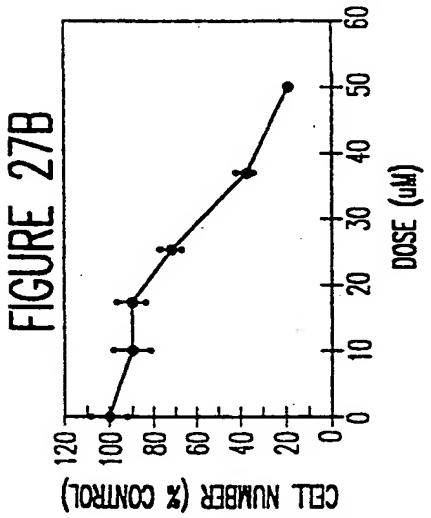


FIGURE 27B
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

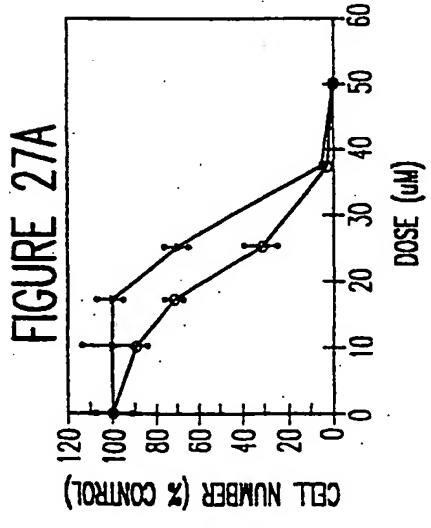


FIGURE 27A
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

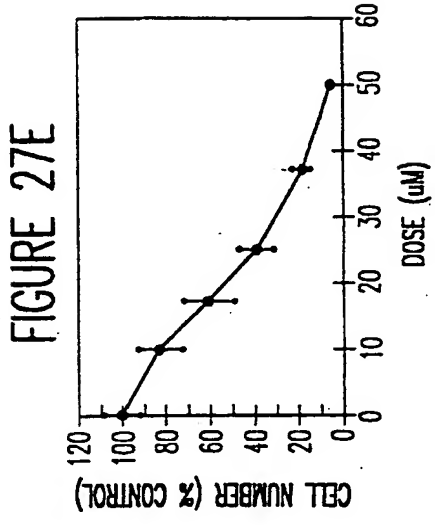


FIGURE 27E
CELL NUMBER (% CONTROL)
DOSE (uM)
0
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80
100
120
0
10
20
30
40
50
60

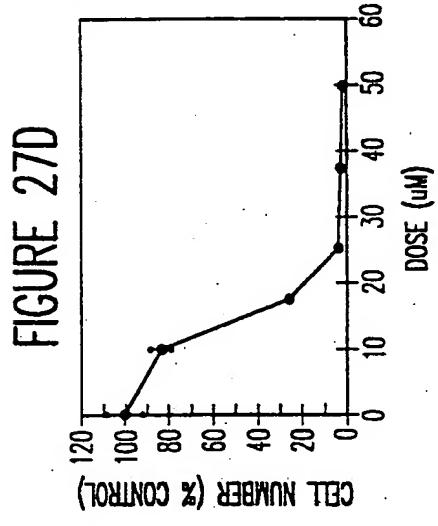


FIGURE 27D
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

FIGURE 28C

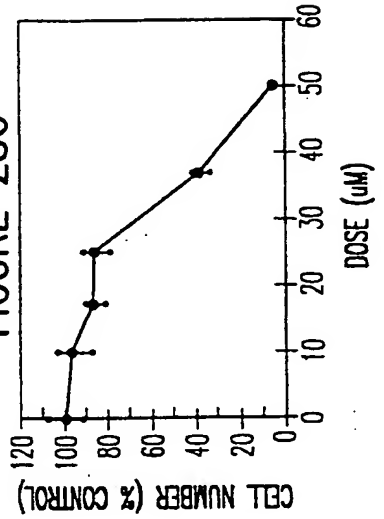


FIGURE 28F

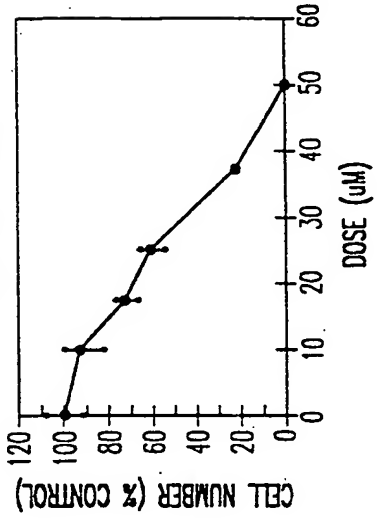


FIGURE 28B

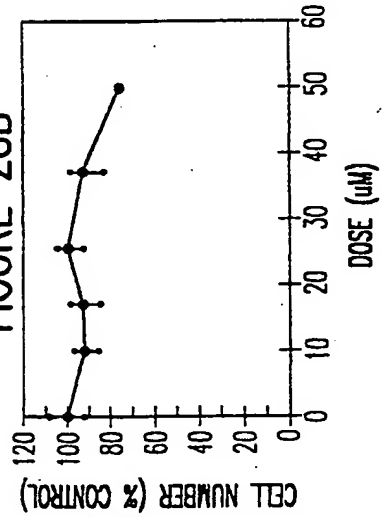


FIGURE 28E

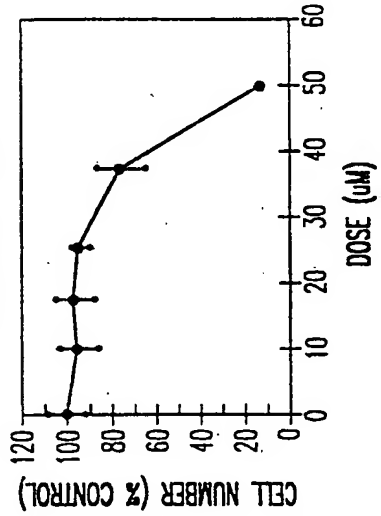


FIGURE 28A

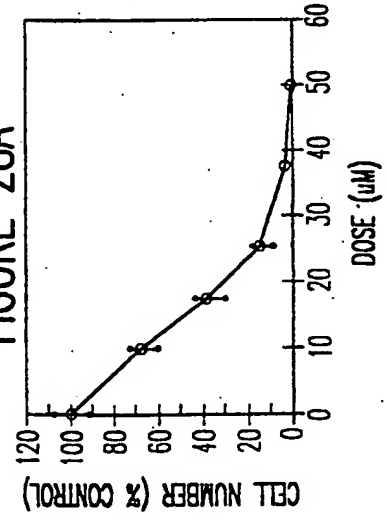


FIGURE 28D

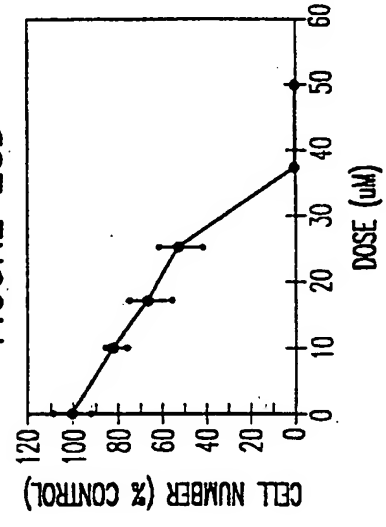


FIGURE 29C

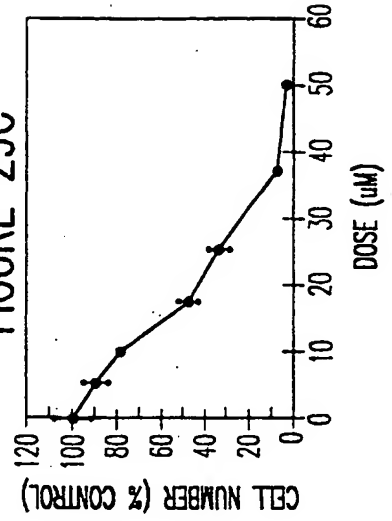


FIGURE 29F

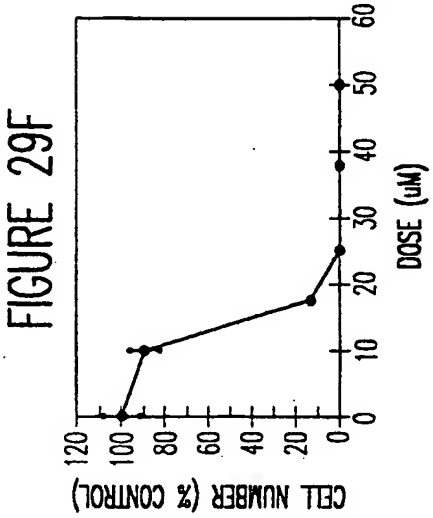


FIGURE 29B

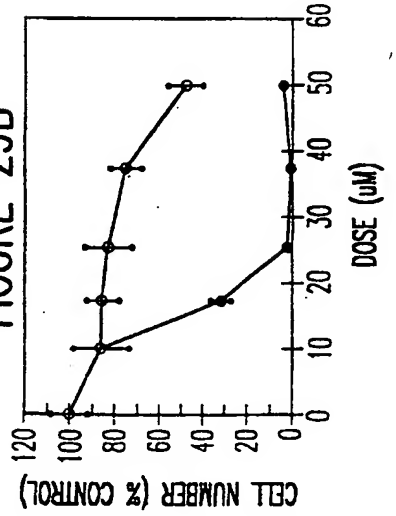


FIGURE 29E

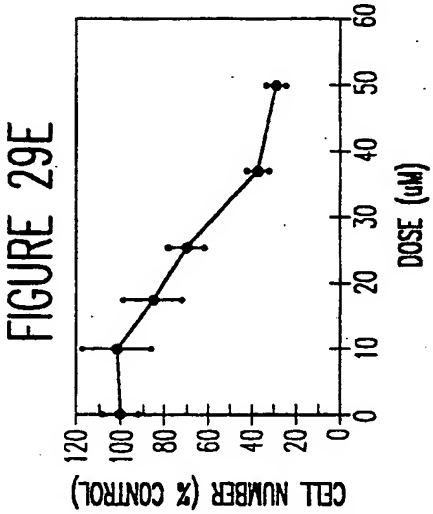


FIGURE 29A

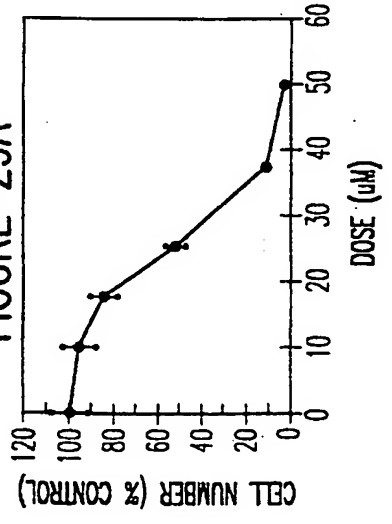
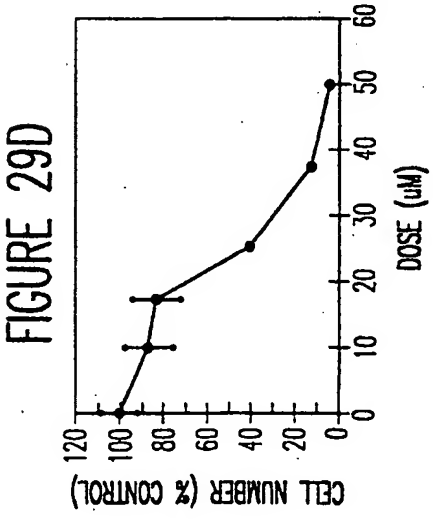


FIGURE 29D



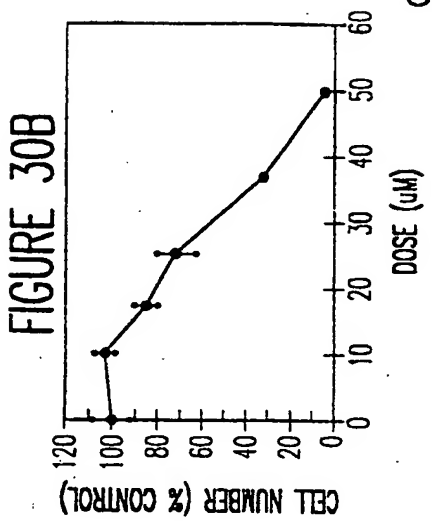


FIGURE 30B
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

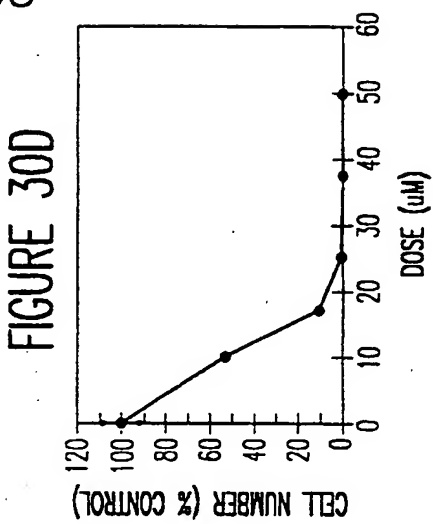


FIGURE 30D
CELL NUMBER (% CONTROL)
DOSE (uM)
0
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40
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80
100
120
0
10
20
30
40
50
60

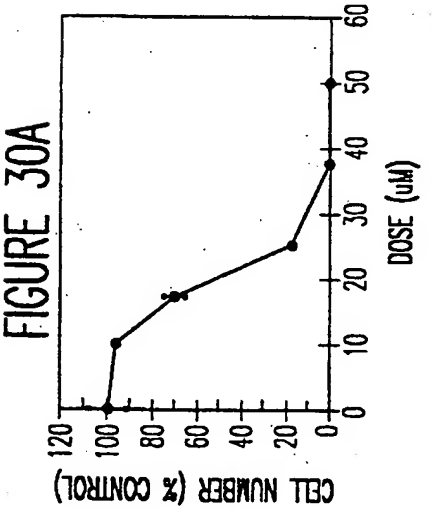
FIGURE 30A
CELL NUMBER (% CONTROL)
DOSE (uM)
0
20
40
60
80
100
120
0
10
20
30
40
50
60

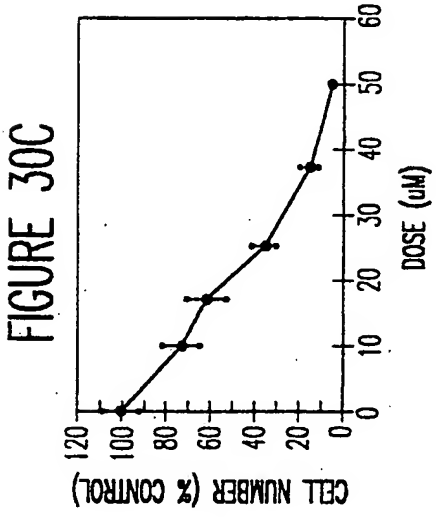
FIGURE 30C
CELL NUMBER (% CONTROL)
DOSE (uM)
0
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60
80
100
120
0
10
20
30
40
50
60

FIGURE 31B

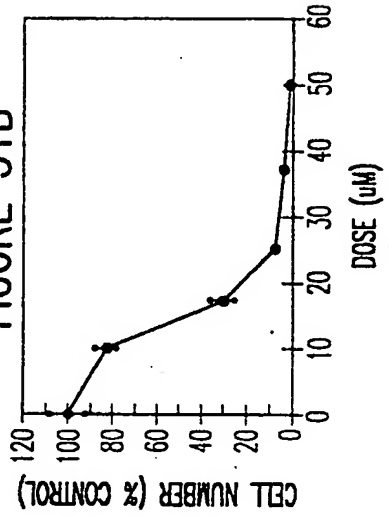

FIGURE 31C

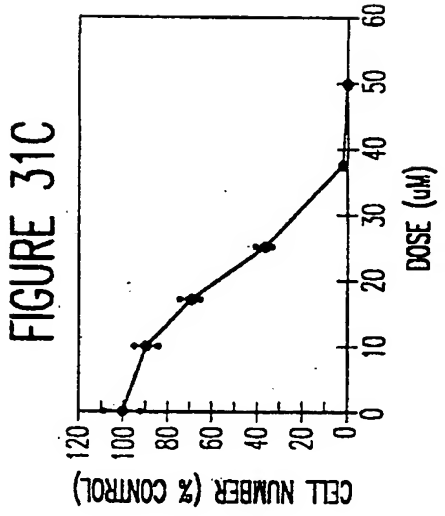

FIGURE 31A

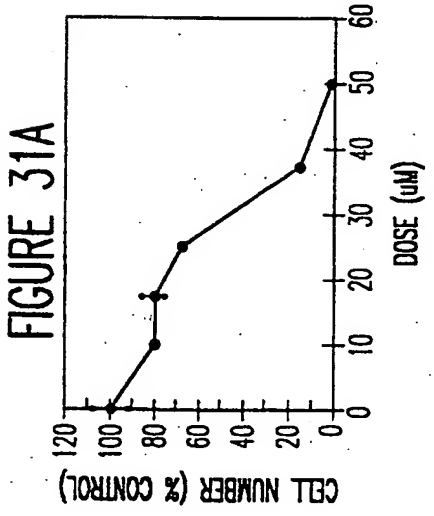

FIGURE 32B

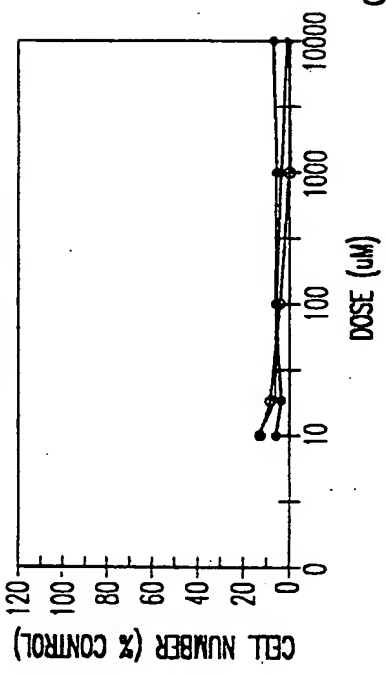

FIGURE 32D

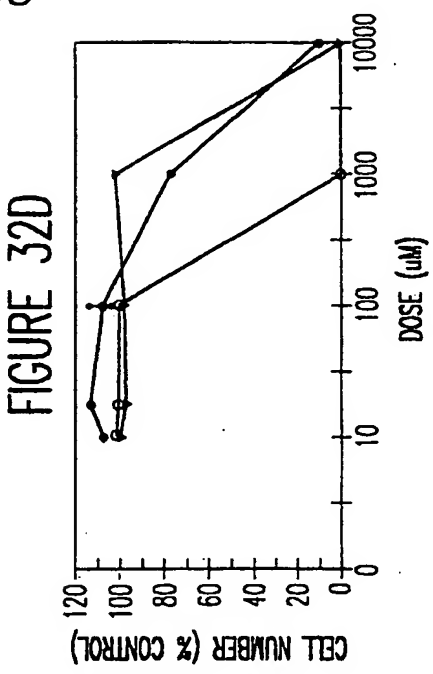

FIGURE 32A

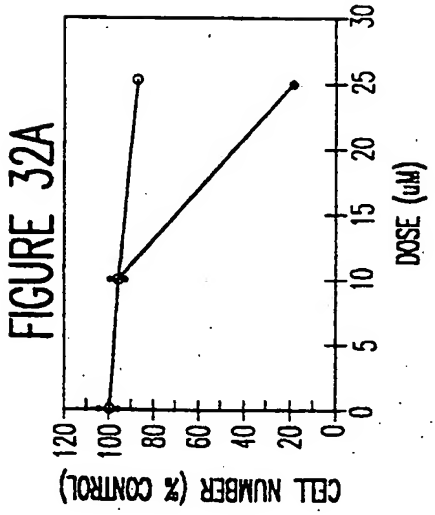

FIGURE 32C

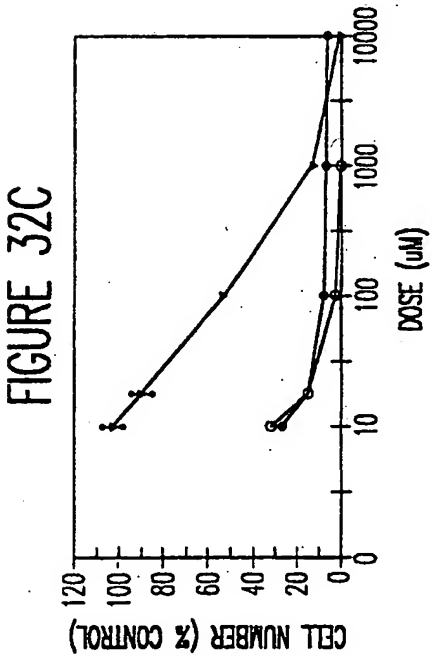

FIGURE 33A

FIGURE 33B

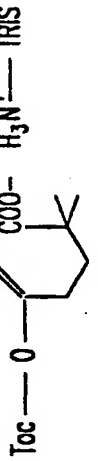

FIGURE 33C

FIGURE 33D

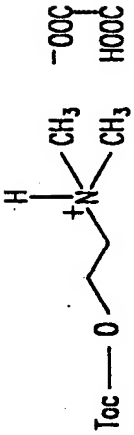

FIGURE 33E